REVIEW ARTICLE

ANTIFOLIC ACIDS AND ANTIPURINES IN CHEMOTHERAPY

By G. M. Timmis, M.Sc., F.R.I.C.

The Chester Beatty Research Institute, The Royal Cancer Hospital, London, S.W.3

In this review an attempt will be made to illustrate the uses and potential uses of antifolic acids and antipurines in chemotherapy. These two types of agent can probably be best defined, in brief, by referring to their ability to inhibit the growth of various organisms; this inhibition can be prevented or reversed by the addition respectively of folic acid (I) or one of the natural purines, usually adenine (II; $R' = NH_2$, $R'' = H$) or guanine (II; $R' = OH$, $R'' = NH_2$). Antagonism experiments in small animals have proved difficult to assess accurately, and the most commonly employed organisms are bacteria, e.g., *Lactobacillus casei*, *Streptococcus faecalis* and *Escherichia coli*. By their use we can ascertain whether the ratio of the potential antagonist to the amount of folic acid, or the purine, required to relieve inhibition remains roughly the same whatever the actual concentrations of the antagonistic substances. In this instance a true competitive antagonism would exist and, when present, constitutes usually the best available evidence for a true folic acid or purine antagonist, since when the antagonism is irreversible or not competitive, we do not know whether the metabolite is essentially involved or not, although the bacteria concerned may normally require folic acid or a purine for growth. It must be emphasised that these simple and ideal conditions seldom, if ever, apply wholly in practice, and they are here used mainly as an illustration of the principle of competitive antagonism and the logical criterion for its presence. For an example of the detailed treatment of this subject the reader may be referred to reference 1. In this review, except where stated, only reversible antagonisms will be considered.

Folic Acid Antagonists

Antifolic acids and antipurines are logically related, since recent work on the biosynthesis of nucleic acids[2–4] reveals the essential role of folinic acid (Citrovorum factor) in the synthesis of their purine constituents. This derivative of folic acid is necessary for the synthesis of glycinamide ribotide, and at a later stage the ring closure of 5-amino-4-iminazole-carbonamide ribotide. Folinic acid (5-formyl-5 : 6 : 7 : 8-tetrahydropteroyl-glutamic acid, III) is formed from folic acid, and it has been shown that, for example, the two powerful antifolic acids, aminopterin (IV; $R' = NH_2$, $R'' = H$) and amethopterin (Methotrexate IV; $R' = NH_2$, $R'' = Me$) inhibit growth by preventing the conversion of folic to folinic acid in various organisms. In these cases also there is probably a direct interference with folinic acid[5–9].

The first chemotherapeutic developments in this field were the successful use of aminopterin and Methotrexate in inducing in some proportion,

remissions in children with acute leukaemia[10]. Rather less useful are the folic acid analogues which contain a hydroxy group in the 4-position, e.g. (IV; R′ = OH, R″ = Me) and they have now fallen out of general clinical use. In general, the clinical effect of these antifolic acids is reflected in experiments with leukaemia in mice[11–15]. The fact that the clinical use of antifolic acids does sooner or later induce a drug resistance is a serious disadvantage. This effect has been alleviated in experimental leukaemias by the combination of two drugs which do not show cross-resistance, e.g., by the use of 6-mercaptopurine (*vide infra*) with Methotrexate[16]. Similar clinical attempts have so far been promising.

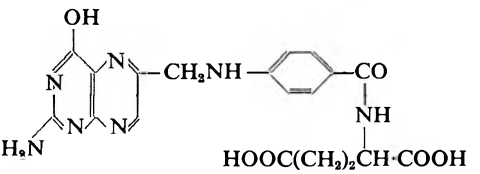
The field of the antifolic acids may still, nevertheless, be worth cultivating further, since Farber[17] has found that drugs of the Methotrexate type have produced impressive improvement in rhabdomyosarcoma, Hodgkin's disease, lymphosarcoma, neuroblastoma and chronic lymphoid leukaemia. Also, Colsky[18] reported improvement in carcinoma of the breast treated with Methotrexate.

Amongst simpler types of folic acid analogues which have yielded, at the least, significant antifolic activity, are the 6:7-substituted diaminopteridines (V). In this type R′ and R″ have been phenyl or substituted phenyl[19,20], R′ phenyl or substituted phenyl and R″ amino[21] or R′ and R″ have both been alkyl or aralkyl, or R′ aryl and R″ alkyl[22–24]. Antifolic acid activity has also been found in more complex types, for example, the naphthopteridine (VI)[25,26] and the indolopteridines (VII; R=alkyl)[22–24]. None of these types has yet yielded compounds of value in leukaemia, but high antimalarial activity has been found in some of the 2:4-diamino-6:7-dialkylpteridines and 2:4-diamino-6-aryl-7-alkylpteridines; e.g., V (R′ = anisyl, R″ = *iso*propyl) which equals proguanil (Paludrine) in activity. But for the emergence of the pyrimidine derivative pyrimethamine (Daraprim, *vide infra*), a drug of wide usefulness clinically would probably have arisen from this series.

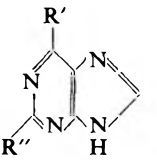
Another potentially interesting type of activity was found both in the 6:7-dialkyl-2:4-diaminopteridines and in the indolopteridines, (e.g., VII; R = Me) where substances with high activity *in vitro* against *Vibrio cholerae* were found.

High antifolic and antimalarial activity has also been found in structures simpler than pteridines, viz., the 2:4-diamino-5-chlorophenyl pyrimidines[27] (e.g., VIII), and the 4:6-diamino-1-chlorophenyl-1:2-dihydrotriazines (e.g., IX)[28–32]. 2:4-Diamino-5-3′:4′-dichlorophenyl-6-methylpyrimidine (VIII; R = Me) resembles aminopterin and Methotrexate in its antifolic acid properties, but it has had only slight success in the treatment of acute leukaemia in children[33] and seems to be inferior to Methotrexate owing to its toxicity. Slight inhibition of growth of the mouse sarcoma 180 has been caused by this type of compound where the methyl group is replaced by hydrogen, ethyl or *n*-amyl groups[34]. Of much greater clinical interest in this group is pyrimethamine[35] (Daraprim, VIII; R = C_2H_5), a widely used antimalarial drug.

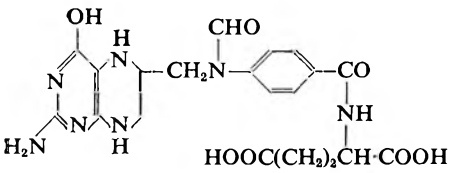
The series of dihydrotriazines (e.g., IX), studied by Modest and Foley and their collaborators, are non-competitive inhibitors of folic acid and



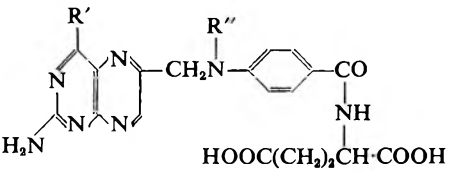
(I)



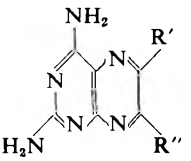
(II)



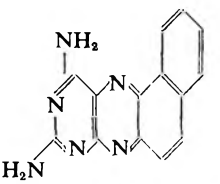
(III)



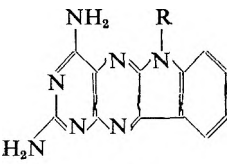
(IV)



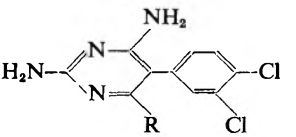
(V)



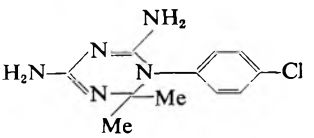
(VI)



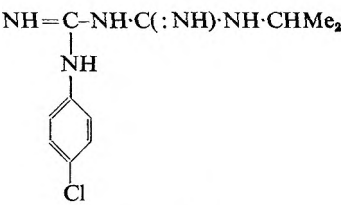
(VII)



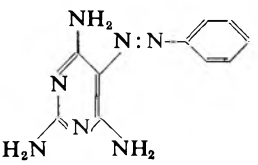
(VIII)



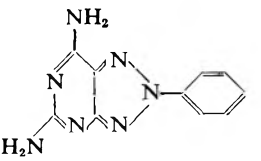
(IX)



(X)



(XI)



(XII)

folinic acid. Although active against experimental leukaemias, clinical experiments with these compounds have been found unpromising possibly because an adequate dose cannot be tolerated[36]. The series has provided many compounds active against experimental malaria[37,38] and experimental tumours[39], but although compound IX is the active metabolite[40] of the widely used antimalarial proguanil (X), and has been found to be more active in experimental animals, its clinical use is at least uncommon, and the same applies to the other members of the series.

Recently discovered antifolic acids are the amino-5-arylazopyrimidines (XI) and the amino-8-aryl-8-azapurines (XII)[1,41]. In neither series is activity high. Of the arylazopyrimidines the 2′:4′-dibromophenyl- and the β-naphthyl-analogues were the most active, having about 1/300th the activity of Methotrexate. The most active of the 8-azapurines was the 2′:4′-dibromophenyl-analogue which had 1/60th of the activity of Methotrexate, tested with *Str. faecalis*. However, antifolic acid activity associated with the 8-azapurine structure is apparently a new observation. The possibility that folic acid fulfils an essential role in the multiplication of viruses has not been neglected. Psittacosis virus (6BC) in chick embryonic tissue was inhibited by aminopterin, Methotrexate and amino-an-fol, which is an analogue of aminopterin (IV; $R' = NH_2$, $R'' = H$) differing only by the substitution of an aspartic acid for the glutamic acid moiety. Since the inhibition caused by the last two compounds was annulled by Citrovorum factor, there is evidence that the effect arises from a direct interference with the folic acid metabolism of the virus[41a].

Antipurines

In the field of antipurines the number of compounds and types of structure discovered with appreciable activity has been much smaller than amongst the antifolic acids. The first example to show any marked activity as an antagonist of adenine in *L. casei* was 2:6-diaminopurine (XIII; $R' = R'' = NH_2$)[41b]. The clinical trials[42] in leukaemia were disappointing, although a significant effect had been observed on a transplanted mouse leukaemia.[43]

At about this time the antiguanine activity of 8-azaguanine (XIV; $R' = OH$, $R'' = NH_2$) was discovered with *Tetrahymena* as the organism,[44] but clinically its effect has been disappointing in trials spread over some six years, and its attempted use seems to have been abandoned.

Of the simple purines, 6-mercaptopurine[45] (XIII; $R' = SH$, $R'' = H$) is by far the most interesting since it is the drug of choice in the treatment of acute leukaemia in adults[46]. Also it is effective in children and in those instances in which resistance has been induced to the antifolic acids and to cortisone. It is also of some value in chronic myeloid leukaemia. 6-Chloropurine (XIII; $R' = Cl$, $R'' = H$) has been tried clinically in chronic and acute leukaemia without marked success[47]. Similarly, trials of 2-amino-6-mercaptopurine (XIII; $R' = SH$, $R'' = NH_2$)[47] and purine itself (XIII, $R' = R'' = H$), which is an adenine antagonist, have revealed, at most, no advantage over 6-mercaptopurine[48].

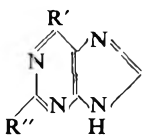
The most complex antipurine of any so far produced is puromycin, 6-dimethylamino-9-(3′-*p*-methoxy-L-phenylalanyl-amino-3′-deoxy-D-ribosyl)purine (XV). This evinces an antiguanine action in *E. coli*[55] and it has been shown that its trypanocidal action in *T. equiperdum* was due to interference with the purine metabolism of the parasite[49]. This trypanocidal action was reversed by adenine and other purines[50]. After showing activity against experimental tumours and leukaemias, all clinical trials in various malignant diseases were disappointing; but effectiveness as an amoebicide was uncovered first in guinea pigs[52] and then in man[53]. When the structure of puromycin was simplified by removal of the *p*-methoxyphenylalanyl residue (at the dotted line shown in XV) the amine obtained, 6-dimethylamino-9-(3′-amino-3′-deoxy-D-ribosyl) purine was similar to puromycin in its activity against experimental tumours and trypanosomiasis. Like puromycin, this compound has not so far shown promise in clinical trials with malignant disease.

New types of purines and azapurines have recently been synthesised as potential antipurines[54] on the hypothesis that cyclic substituents in the 9 position might be of value since they bear a spacial relation to the furanose ring (cf. XVI) which appears, in the form of the phosphate ester, as a stage in the normal biosynthesis of the hypoxanthine and presumably other purine moieties in ribonucleic acid, and possibly as the deoxy form in deoxyribonucleic acid.

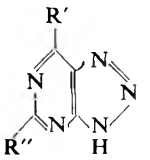
The formulae XVII to XIX illustrate four active compounds, all of them showing essentially an antiguanine type of activity when tested with *E. coli*[55]. The azapurine (XVII) is about three times as active in this respect as is 6-mercaptopurine. The other compounds are less active; XVIII (R = Me or H) also show a less competitive type of action than does XVII, and XIX has a less competitive kind of inhibition than XVIII.

Of purine analogues, a little more removed from purine in structure, a series of thiazolopyrimidines (3:4:6-triaza-1-thiaindenes) (e.g., XX) and a series of pyrazolopyrimidines (1:2:5:7-tetrazaindenes) (XXI) have yielded active compounds. In the 1:2:5:7-tetrazaindene series[57] activity against those experimental tumours usually susceptible to the action of antipurines has been found[58]. In *E. coli*, XXI (R = Me) is about three times weaker than 6-mercaptopurine as an antiguanine, and had very little adenine activity[59]. Very recently, partially competitive antiguanine activity has been found in the 8-thiapurine derivative XXII[60]. Also, in the 2-azapurine series, 2-azaadenine (XXIII; R = NH_2) and 2-azahypoxanthine (XXIII; R = OH) are antipurines[62].

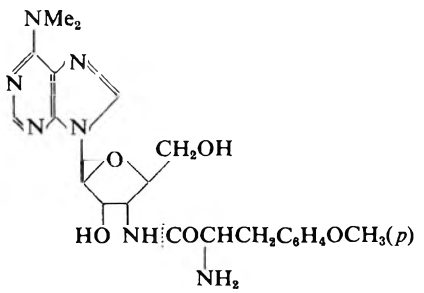
Benziminazole (XXIV) and its derivatives and benzthiazole (XXV) and benztriazole (XXVI) are the antipurines furthest removed from a structural analogy with purine. Benziminazole was first found to have anti-adenine activity in various organisms[63], and an antiguanine activity in *E. coli* was found later[55]. Benzthiazole and benztriazole were similarly found to be weak antiguanines[55]. Comparison of benztriazole with 8-azapurine (XXVII)[1] showed that the latter had a similar weak, mainly antiguanine, type of activity. Thus, in this instance, substitution of the



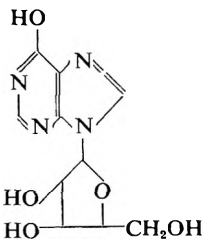
(XIII)



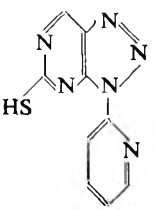
(XIV)



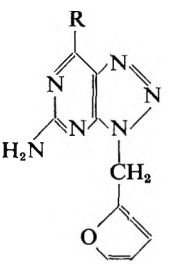
(XV)



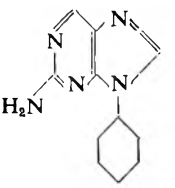
(XVI)



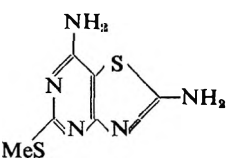
(XVII)



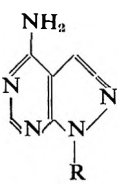
(XVIII)



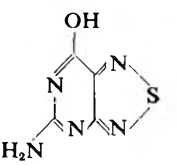
(XIX)



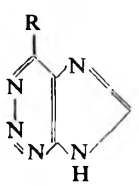
(XX)



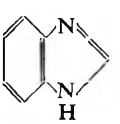
(XXI)



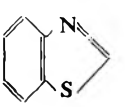
(XXII)



(XXIII)



(XXIV)



(XXV)

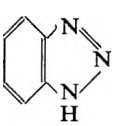
benzene ring by the pyrimidine ring, which is chemically very different and resembles much more a 2:4-dinitrobenzene ring, made little difference.

Antipurines and Viruses

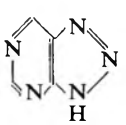
It is convenient at this point to consider the effect of antipurines on viruses. 8-Azaguanine (XIV; R′ = OH, R″ = NH_2) has been extensively investigated and causes a marked inhibition of systemic development of lucerne mosaic virus in *Nicotiana glutinosa*, and tobacco and cucumber mosaic virus in cucumbers and *Nicotiana sp.*[64]. 8-Azaadenine (XIV; R′ = NH_2, R″ = H) has also some inhibitory effect on lucerne mosaic, cucumber mosaic and tobacco mosaic viruses. The virus inhibition by 8-azaguanine can be reversed by spraying the plants with adenine, guanine or hypoxanthine indicating an antipurine mechanism for the inhibition. Furthermore, 8-azaguanine is incorporated into the virus nucleic acid[65] and the infectivity of the virus was markedly reduced[65].

Vaccinia virus in tissue culture was also inhibited by 2:6-dichloro-8-hydroxypurine (XXVIII; R = R′ = Cl, R″ = H, R‴ = OH), 2:6:8-trichloropurine (XXVIII; R = R′ = R‴ = Cl, R″ = H) and 2:6-dichloro-7-methylpurine (XXVIII; R = R′ = Cl, R″ = Me, R‴ = H), but the inhibition was not reversed by normal purines[66].

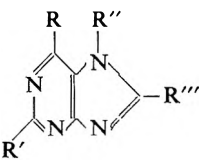
2:6-Diaminopurine (XXVIII; R = R′ = NH_2, R″ = R‴ = H) was more active and its action was reversed by adenine and hypoxanthine[66]. Benziminazole[67–70] and derivatives of benziminazole have, however, led to more interesting results than purine derivatives or close analogues, and a detailed and systematic exploration of structure-activity relations in this field has been carried out[71,72]. By the attachment of a ribose moiety to the most active of the methyl and chlorine substituted benziminazoles, even more active compounds were obtained of which 4:5:6-trichloro-1-β-D-ribofuranosylbenziminazole (XXIX) was the best, being 760 times more active than benziminazole in the inhibition of Lee



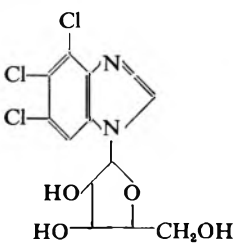
(XXVI)



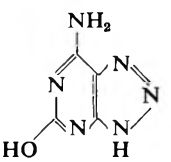
(XXVII)



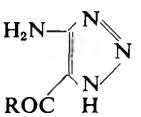
(XXVIII)



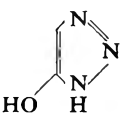
(XXIX)



(XXX)



(XXXI)



(XXXII)

influenza virus in chorio-allantoic membrane tissue culture[73,74]. Although no unequivocal evidence was obtained that the mechanism of action involved an antipurine or antinucleoside effect, it was thought, after elimination of other possible mechanisms, that the action is intracellular and involves disturbance of the metabolism of ribosides. It must be added that no purine analogue or benziminazole derivative has so far been successfully used clinically in a virus disease.

Mechanism of Action of Antipurines

A great deal of work has been directed towards elucidating the essential mechanism of action by which the antipurines inhibit growth. This may be conveniently divided into the conception of the antipurine being incorporated into the nucleic acid molecule, thus producing dysfunction, and the concept that the agent interferes initially with some enzyme or co-enzyme concerned in some way with growth or interferes with the synthesis of nucleic acid from preformed purines. The evidence suggests that any of these mechanisms can operate according to the particular antipurine and also to the organism involved.

8-Azaguanine, and also 8-azaxanthine, are incorporated principally into the ribonucleic acid (RNA) of *B. cereus*, the azaxanthine being converted into azaguanine[75]. Incorporation into the deoxynucleic acid (DNA) was only slight. In both instances marked inhibition of growth occurred. Furthermore, comparison of the effects of 8-azaguanine, 8-azaadenine, 8-azahypoxanthine, 8-aza*iso*guanine (XXX), 4-amino-triazole-5-carbonamide (XXXI; R = NH_2), 4-aminotriazole-5-carboxylic acid (XXXI; R = OH) and 5-hydroxytriazole (XXXII) on tobacco mosaic virus and *E. coli* showed that where growth inhibition occurred, so did incorporation into RNA and where there was no inhibition RNA was not incorporated[76]. Since the evidence is strong that RNA plays, at least, a much bigger part in protein synthesis than does DNA, the comparison of the actions of these closely related compounds provides good evidence for associating incorporation with growth inhibition. Again with 8-azaguanine in *E. coli*, inhibition of growth does not begin until one to two generation times after the inhibitor has been added. The time lag in the onset of growth inhibition, which does not occur with other bacterial growth inhibitors, suggested that time was necessary for the accumulation of damaged and ineffective RNA before an effect on growth was evident. On the other hand, a comparison with the inhibition of growth of *B. cereus* by azaguanine shows that for a roughly equivalent amount of inhibition much less azaguanine is incorporated into the RNA[77]. This result suggests that another mechanism may be playing a larger part in inhibiting *B. cereus* than is seen with *E. coli*. This idea has been confirmed by experiments with 8-azaguanine (labelled with ^{14}C at the carbon in the 4-position) and *B. cereus*, from which it was concluded that inhibition was produced either by the incorporation of the inhibitor into a particularly sensitive portion of the nucleic acid or by interference with some co-factor[78]. Study of the effect of 6-mercapto-purine on the growth of *E. coli* showed that at low concentrations of the

inhibitor which produced inhibition, no effect on nucleic acid biosynthesis could be detected. Mechanisms other than an effect on nucleic acid must therefore be sought[79] and the inhibitors may well interfere in some way with co-enzyme A[80,81], pantothenic acid[81] or with diphosphopyridine nucleotide (DPN)[83]. In this connection it should be noted that co-enzyme A contains an adenine moiety linked via ribose and phosphoric acid to the pantothenic acid amide of β-mercaptoethylamine, whilst DPN also contains an adenine residue linked through ribose and phosphate to nicotinamide.

Hitherto there has been a tendency, when attempting to design new drugs intended to improve on the results so far obtained with antipurines, to consider only the structures of the established links in the chain of nucleic acid synthesis, or smaller moieties, e.g., purines, derivable therefrom, and then to think of analogous structures which might interfere with their function. A probably important consequence of the recent evidence on the mechanism of the inhibitory action of 8-azaguanine and 6-mercaptopurine will be to focus more attention on the possibility of interfering with the action of the two co-factors mentioned above and also with others.

The relation of the action of the sex hormones to the metabolism of folic acid and purines has received very little attention except from the work of Hertz and Tullner[84]. They found that the growth of chick oviduct, a tissue dependent on oestrogens, is much stimulated by administration of stilboestrol and folic acid and that this effect is competitively reversed by aminopterin, Methotrexate and 4-aminopteroylaspartic acid (amino-an-fol.). Similar results were obtained with the uterus of the sexually immature rat. Similarly an antagonism between an antipurine (2:6-diaminopurine) and adenine was demonstrated. Analogous antagonisms were observed between certain steroid hormones[84,85], and the results of this and further work might well help to explain, for example, why both antifolic acids and cortisone are beneficial in acute leukaemia.

Summary

A review of the field of antifolic acids and antipurines made with the intention of illustrating their use, or potential use, in chemotherapy, reveals drugs useful, but by no means ideal, for the palliative treatment of leukaemia, and the discovery of one valuable antimalarial drug, which is an antifolic acid. There is also evidence that an antipurine mechanism can be involved in trypanocidal and also in antiviral action, but successful clinical applications of these leads have not yet been achieved. Again, there is a preliminary indication that antifolic acids might perhaps be of some value as antiviral agents and here, as with the antipurines, further work will doubtless be stimulated by the enormous importance of a successful chemotherapy of virus diseases, which is yet to be achieved.

References

1. Timmis, Felton, Collier and Huskinson, *J. Pharm. Pharmacol.*, 1957, **9**, 46.
2. Schulman and Buchanan, *J. biol. Chem.*, 1952, **196**, 513.
3. Greenberg, *Fed. Proc.*, 1953, **12**, 651.

4. Hartman, Levenberg and Buchanan, *J. Amer. chem. Soc.*, 1955, **77**, 501.
5. Nichol and Welch, *Proc. Soc. exp. Biol., N.Y.*, 1950, **74**, 52, 403.
6. Sauberlich, *J. biol. Chem.*, 1949, **181**, 467.
7. Hendlin, Koditschek and Soars, *J. Bact.*, 1953, **65**, 466.
8. Broquist, Kohler, Hutchison and Burchenal, *J. biol. Chem.*, 1953, **202**, 59.
9. Nichol, Zakrzewski and Welch, *Proc. Soc. exp. Biol., N.Y.*, 1953, **83**, 272.
10. Farber, *Blood*, 1949, **4**, 160.
11. Weir, Welch and Heinle, *Proc. Soc. exp. Biol., N.Y.*, 1949, **71**, 107.
12. Kirschbaum, Geisse, Judd and Meyer, *Cancer Res.*, 1950, **10**, 762.
13. Law, Dunn, Boyle and Miller, *J. Nat. Cancer Inst.*, 1949, **10**, 179.
14. Burchenal, Johnston, Burchenal, Kushida, Robinson and Stock, *Proc. Soc. exp. Biol., N.Y.*, 1949, **71**, 381.
15. Burchenal, Burchenal, Kushida, Johnston and Williams, *Cancer*, 1949, **2**, 113.
16. Skipper, Thomson and Bell, *Cancer Res.*, 1954, **14**, 503.
17. Farber, Toch, Sears and Pinkel, *Advances in Cancer Research*, Academic Press, New York, 1956, **4**, p. 39.
18. Colsky, *Chemistry and Biology of Pteridines*, J. and A. Churchill, London, 1954, p. 413.
19. Daniel, Norris, Scott and Heuser, *J. biol. Chem.*, 1947, **169**, 689.
20. Daniel and Norris, *ibid.*, 1947, **170**, 747.
21. Spickett and Timmis, *J. chem. Soc.*, 1954, 2887.
22. Campbell, Dunsmuir and Fitzgerald, *ibid.*, 1950, 2743.
23. Campbell, Collier, Dunsmuir and Fitzgerald, British Patent No. 666,788/1952.
24. Dunsmuir, British Patent Application No. 6514/1954.
25. Felton and Timmis, *J. chem. Soc.*, 1954, 2881.
26. Collier, 1954, unpublished.
27. Hitchings, Falco, Vanderwerff, Russell and Elion, *J. biol. Chem.*, 1952, **199**, 43.
28. Modest, Foley, Pechet and Farber, *J. Amer. chem. Soc.*, 1952, **74**, 855.
29. Modest, Farber and Foley, *Proc. Amer. Assoc. Cancer Res.*, 1954, **1**, 33.
30. Modest, *J. org. Chem.*, 1956, **21**, 1.
31. Foley, *Proc. Soc. exp. Biol., N.Y.*, 1953, **83**, 733, 740.
32. Carrington, Crowther and Stacey, *J. chem. Soc.*, 1954, 1017.
33. Murphy, Ellison, Karnofsky and Burchenal, *J. clin. Invest.*, 1954, **33**, 1388.
34. Clarke, Buckley, Sternberg, Stock, Rhoads and Hitchings, *Cancer Res.*, 1952, **12**, 255.
35. Falco, Goodwin, Hitchings, Rollo and Russell, *Brit. J. Pharmacol.*, 1951, **6**, 185.
36. Farber, Toch, Sears and Pinkel, *Advances in Cancer Research*, Academic Press, New York, 1956, **4**, p. 40.
37. Hewitt, Wallace, Gumble, White and Williams, *Amer. J. trop. Med. and Hyg.*, 1954, **3**, 225.
38. Schmidt, Loo, Fradkin and Hughes, *Proc. Soc. exp. Biol., N.Y.*, 1952, **80**, 367.
39. Farber, Appleton, Downing, Heald, King and Toch, *Cancer*, 1953, **6**, 135.
40. Carrington, Crowther, Davey, Levi and Rose, *Nature, Lond.*, 1951, **168**, 1080.
41. Modest, Schlein and Foley, *J. Pharm. Pharmacol.*, 1957, **9**, 68.

41*a*. Morgan, *J. exp. Med.*, 1952, **95**, 269.

41*b*. Hitchings, Elion, Vanderwerff and Falco, *J. biol. Chem.*, 1948, **174**, 765.

42. Burchenal, Karnofsky, Kingsley-Pillers, Southam, Myers, Escher, Craven, Dargeon and Rhoads, *Cancer*, 1951, **4**, 459.
43. Burchenal, Bendick, Brown, Elion, Hitchings, Rhoads and Stock, *ibid.*, 1949, **2**, 119.
44. Kidder and Dewey, *J. biol. Chem.*, 1949, **179**, 181.
45. Elion, Burgi and Hitchings, *J. Amer. chem. Soc.*, 1952, **74**, 411.
46. Hall, Richards, Willett and Feichtmeir, *Ann. N.Y. Acad. Sci.*, 1954, **60**, 374.
47. Murphy, Tan, Ellison, Karnofsky and Burchenal, *Proc. Amer. Assoc. Cancer Res.*, 1955, **2**, 36.
48. Farber, *Advances in Cancer Research*, Academic Press, New York, 1956, **4**, p. 43.
49. Agosin and Brand, *Antibiotics and Chemotherapy*, 1954, 4, 624.
50. Hewitt, Gumble, Wallace and Williams, *ibid.*, 1954, **4**, 1222.
51. Wright, Dolgolpol, Logan, Prigot and Wright, *Proc. Amer. Assoc. Cancer Res.*, 1955, **2**, 55.
52. Taylor, Sherman and Bond, *J. Amer. chem. Soc.*, 1954, **76**, 4497.
53. Faiguenbaum and Alba, *Boletin chileno de parisitologia*, 1954, **9**, 94.
54. Timmis, Cooke and Spickett, *The Chemistry and Biology of Purines*, J. and A. Churchill, London, 1957, p. 148.

55. Collier and Huskinson, *The Chemistry and Biology of Purines*, J. and A. Churchill, London, 1957, p. 134.
56. Hitchings, Elion and Sherwood, *Fed. Proc.*, 1950, **9**, 185.
57. Robins, *J. Amer. chem. Soc.*, 1956, **78**, 784.
58. Skipper, Robins and Thomson, *Proc. Soc. exp. Biol., N.Y.*, 1955, **89**, 594.
59. Collier and Huskinson, 1956, unpublished.
60. Timmis, *J. chem. Soc.*, 1957, in the press.
61. Woolley and Shaw, *J. biol. Chem.*, 1951, **189**, 401.
62. Shaw and Woolley, *ibid.*, 1952, **194**, 640.
63. Woolley, *ibid.*, 1944, **152**, 225.
64. Matthews, *J. gen. Microbiol.*, 1953, **8**, 277.
65. Matthews, *ibid.*, 1954, **10**, 521.
66. Thompson, Price, Minton, Elion and Hitchings, *J. Immunol.*, 1950, **65**, 529.
67. Brown, *ibid.*, 1952, **69**, 441.
68. Thompson, *J. Bact.*, 1946, **51**, 629.
69. Thompson, *J. Immunol.*, 1947, **55**, 345.
70. Morgan, *J. exp. Med.*, 1952, **95**, 277.
71. Tamm, Folkers, Shunk, Heyl and Horsfall, *ibid.*, 1953, **98**, 245.
72. Tamm, Folkers, Shunk and Horsfall, *ibid.*, 1954, **99**, 227.
73. Tamm, *Science*, 1954, **120**, 847.
74. Tamm, Folkers and Shunk, *J. Immunol.*, 1956, **72**, 54.
75. Mathews and Smith, *Nature, Lond.*, 1956, **177**, 271.
76. Mathews and Smith, *Biochem. J.*, 1954, **57**, vi.
77. Mathews, *Chemistry and Biology of Purines*, J. and A. Churchill, London, 1957, p. 270.
78. Mandel, *J. biol. Chem.*, 1957, in the press.
79. Bolton and Mandel, *J. Pharmacol.*, 1957, in the press.
80. Biesele, *Ann. N.Y. Acad. Sci.*, 1954, **60**, 228.
81. Garattini, Mopurgo and Passerini, *Giorn. ital. Chemterap.*, 1955, **2**, 29.
82. Caliari and Garattini, *ibid.*, 1955, **2**, 23.
83. Kaplan, Goldin, Humphreys, Ciotti and Stolzenbach, *J. biol. Chem.*, 1956, **219**, 287.
84. Hertz and Tullner, *Ann. N.Y. Acad. Sci.*, 1950, **52**, Art. 8, 1260.
85. Hertz, *Antimetabolites and Cancer*, American Association for the Advancement of Science, Washington, 1955, p. 253.

RESEARCH PAPERS

PAPER CHROMATOGRAPHY OF SOME CARDIAC GLYCOSIDES AND THEIR DERIVATIVES

BY JEANETTE HARRISON AND S. E. WRIGHT

From the Pharmacy Department, University of Sydney, Sydney, Australia

Received July 2, 1956

SEVERAL paper chromatographic systems have been used successfully for the separation of cardiac glycosides[1] and some information has been obtained about the relation between the chemical structure of several glycosides and their chromatographic behaviour. Schindler and Reichstein[2] chromatographed a number of aglycones and their glycosides on formamide impregnated paper and showed the importance of an increase in oxygen content of the aglycone on the rate of travel. Heftmann and Levant[3], also using formamide paper, demonstrated the influence of the degree of oxidation of the side chain at C(10) on the rate of travel of glycosides of the strophanthidin group. They also found that acetylation of the hydroxyl groups of the aglycone increased the rate of travel, presumably because the polarity of the molecule had been reduced.

Tschesche, Grimmer, and Seehofer[4] sought to overcome the adsorption effects of the paper and obtain a true partition effect by using a reversed phase system of chromatography in which the organic phase was applied to the paper, and the chromatogram developed with the aqueous phase. Using an amyl alcohol—water mixture, and also different mixtures of *n*-octanol, amyl alcohol, water, and formamide, very consistent R_F values were obtained and although adsorption effects were not eliminated, they appeared to be reduced to a minimum.

Owing to the apparent close approach to a true partition effect given by these systems, we have now used similar solvent mixtures to examine the effect on the chromatographic behaviour of structural differences in a number of digitalis glycosides and their derivatives. The movement of several of the substances on formamide paper has also been investigated.

EXPERIMENTAL

Materials

Ethylhexanol b.p., 183–184°; amyl alcohol (May and Baker) b.p., 128–132°; chloroform purified by passage through silica gel column; benzene b.p., 80°; formamide (Light and Company); filter paper, Whatman No. 4.

Chromatography Systems

[All solvents measured by volume.]

System I: ethyl hexanol–amyl alcohol–water–formamide (6:2:8:2).

System II: ethyl hexanol–amyl alcohol–water–formamide (6:2:1:4).

System III: amyl alcohol–water (1 : 1).

System IV: Chloroform–benzene (78 : 12) saturated with formamide; formamide impregnated paper.

The solvents for Systems I, II, and III were shaken together and allowed to separate. The organic phase was sprayed onto filter paper, 10 cm. × 30 cm., so that it was just saturated. Four substances (20–40 μg. in solution) were applied to the starting line 5 cm. from the end of the paper. The chromatograms were suspended in a glass tank and developed with the aqueous phase by the descending method at 18–24°, until the solvent front had advanced to 28 cm. from the starting line. The top of the chromatogram was raised out of the aqueous phase, allowed to drain for 10 minutes to prevent streaking, and then the chromatogram was dried at 100°. The substances were located by spraying lightly with a 33 per cent solution of trichloroacetic acid in chloroform containing one drop of 100 volume hydrogen peroxide in each 10 ml.[5] The chromatogram was heated in an oven at 110° for 10 minutes and examined under ultra-violet light. Substances which could not be detected readily by this reagent were revealed by treating the papers with 5 per cent *m*-dinitrobenzene in benzene, followed by 20 per cent aqueous sodium hydroxide.

For System IV, filter paper strips 1 cm. × 30 cm. were dipped in a freshly prepared solution of 25 per cent formamide in methanol, and excess solution removed by pressing lightly between filter paper. The substances in solution were applied to the strips along a starting line, 5 cm. from one end. The chromatograms were then developed in a horizontal tank for seven hours at 32°. The substances were detected on the paper with alkaline *m*-dinitrobenzene as described above.

At least two consistent R_F values were obtained for each substance when using the first three systems. To check the consistency of the results, each chromatogram included a standard glycoside. Digoxin was used in System I, digitoxin in System II, lanatoside C in System III, and digoxin in System IV.

TABLE I

R_F VALUES OF COMPOUNDS OF A, B, AND C SERIES OF DIGITALIS GLYCOSIDES

Solvent system	R_F Values		
	I	II	III
Digitoxigenin	0·10	0·60	—
Gitoxigenin	0·27	—	—
Digoxigenin	0·46	—	—
Digitoxin	0·12	0·65	—
Gitoxin	0·29	—	—
Digoxin	0·47	—	—
Lanatoside A	0·61	—	—
Lanatoside B	0·80	—	0·14
Lanatoside C	0·89	—	0·34
Deacetyl lanatoside A	0·69	—	0·08
Deacetyl lanatoside B	—	—	0·24
Deacetyl lanatoside C	—	—	0·44

RESULTS

Solvent System I was found to be suitable for most of the substances and was evidently of fairly wide application, whereas System II was only useful for those compounds of low water solubility, and System III was used for the more water soluble lanatosides. In Table I, a comparison is made of the results obtained for analagous compounds belonging to the

TABLE II

R_F VALUES OF GLYCOSIDES OF THE A SERIES AND DERIVATIVES

Solvent system	R_F Values		
	I	II	III
Acetyldigitoxigenin	0·05	0·35	—
β-Anhydrodigitoxigenin	0·08	0·37	—
Digitoxigenin	0·10	0·60	—
Dihydrodigitoxigenin	0·14	0·61	—
3-*epi*-Digitoxigenin	—	0·62	—
Digitoxin	0·12	0·65	—
Digitoxigenone	0·22	0·75	—
Lanatoside A	0·61	—	—
Deacetyl lanatoside A	0·69	—	0·08

TABLE III

R_F VALUES OF GLYCOSIDES OF THE B SERIES AND DERIVATIVES

Solvent system	R_F Values		
	I	II	III
Gitoxigenin	0·27	—	—
Gitoxin	0·29	—	—
Lanatoside B	0·80	—	0·14
Deacetyl lanatoside B	—	—	0·24

TABLE IV

R_F VALUES OF GLYCOSIDES OF THE C SERIES AND DERIVATIVES

Solvent system	R_F Values		
	I	II	III
$\Delta^{14:16}$ Anhydrodigoxigenin	0·07	0·51	—
14-Deoxydigoxigenin*	0·11	0·53	—
Diacetyldigoxigenin	0·12	0·57	—
12-Acetyldigoxigenin	0·23	0·76	—
Acetyldigoxin	0·34	—	—
Digoxigenin	0·46	—	—
Digoxin	0·47	—	—
Dihydrodigoxigenin	0·48	—	—
Dihydrodigoxin*	0·55	—	—
Digoxigenin-3-one*	0·58	—	—
Lanatoside C	0·89	—	0·34
Deacetyl lanatoside C	—	—	0·44

*Description to be published.

three digitalis glycoside series; A (derivatives of digitoxigenin); B (derivatives of gitoxigenin); C (derivatives of digoxigenin). R_F values of different members of the three series and their derivatives are compared in Tables II, III, and IV.

Owing to the nature of the formamide system, consistent R_F values were not readily obtained, but it was possible to observe the order in

TABLE V

Comparison of digitalis glycosides and derivatives of the A, B, and C series on system IV

Sequence from slow to fast moving substances.

(i) *A Series*	(ii) *B Series*	(iii) *C Series*
Deacetyl lanatoside A	Deacetyl lanatoside B	Deacetyl lanatoside C
Lanatoside A	Lanatoside B	Lanatoside C
Digitoxigenin	Gitoxigenin	Digoxigenin
Anhydrodigitoxigenin and digitoxigenin at solvent front	Gitoxin	Digoxigenin-3-one
		Digoxin
		β-Anhydrodigoxigenin
		Acetyldigoxin
		14-Deoxydigoxigenin, 12-acetyldigoxigenin, and diacetyldigoxigenin at solvent front

which the compounds travelled. This has been tabulated for each of the three series in Table V.

Discussion

Tables I–IV illustrate the effect of the presence and positions of hydroxyl groups in the genins on the R_F values of these substances. A gradual increase in R_F values can be observed for both glycosides and genins passing from the A series through the B series to the C series (Table I). This transition has been noted on other chromatographic systems[6,7] and would appear to be due to an increase in the polarity of the molecules. The presence of a second hydroxyl group in the genin would be expected to produce an increase in R_F values in the reversed phase systems used in this study, but a hydroxyl group at position C(12) (digoxigenin and its glycosides) appears to contribute more to this effect than does a C(16) hydroxyl group (gitoxigenin and its glycosides). The β orientated lactone ring at C(17) would appear to make the C(16) hydroxyl group which is also β orientated[7] less available for hydrogen bonding.

From a comparison of the R_F values of digoxigenin, digitoxigenin and the corresponding $\Delta^{14:15}$ anhydro compounds (Tables II and IV), it appears that the loss of the C(14) β hydroxyl group in the formation of the anhydrogenin produces a definite decrease in mobility, despite the fact that this hydroxyl group is hindered by the neighbouring β-methyl group at position C(13). The effect of the loss of this C(14) β hydroxyl group is also shown in 14-deoxydigoxigenin which has a much lower R_F value than digoxigenin. The C(14) deoxy compound, which still retains hydroxyl groups at C(3) and C(12), has an R_F value very close to that of digitoxigenin (hydroxyl groups at C(3) and C(14)). This would indicate that the hydroxyl groups at C(12) and C(14) contribute equally to the rate of movement and possibly to the polarity of the compounds.

A change in the orientation of the C(3) hydroxyl group from β to α (*epi*-digitoxigenin, Table II), has little effect upon the R_F value, and *epi*-digitoxigenin could not be separated from digitoxigenin by the use of these solvent systems.

Acetylation of hydroxyl groups in the genin produces a consistent lowering of R_F values which is in accord with a decrease in polarity. Thus, digoxigenin, 12-acetyldigoxigenin and 3 : 12-diacetyldigoxigenin follow one

another in that order. A similar effect was noted by Heftmann and Levant for acetates of glycosides and genins on formamide impregnated paper[3].

A comparison of the R_F values of the deacetyl lanatosides A, B, and C, with the corresponding desgluco-derivatives—digitoxin, gitoxin, and digoxin—shows that the glucose residue has an important effect upon the rate of travel of the molecule. It would appear that the four free hydroxyl groups of the glucose molecule contribute a good deal more to the R_F value than the one free hydroxyl group of each digitoxose residue. The digitoxose hydroxyl group appears to be hindered as there is very little difference in R_F values between the desglucoglycosides—digitoxin, gitoxin, and digoxin—and their respective genins. Evidently, for these systems at least, the effect of a hydroxyl group at position C(3) in the genin is about equal to the total effect of the three digitoxose residues. This does not occur in paper chromatographic systems in which water is the stationary phase, as the genins tend to follow the solvent front in systems designed to separate the desglucoglycosides. Acetylation of hydroxyl groups on the sugars of the glycosides has a similar effect on the R_F value to acetylation of hydroxyl groups on the genin. Thus acetyldigoxin follows digoxin, and lanatosides A, B, and C, follow the corresponding deacetyl lanatosides.

As System IV is the reverse of the previous ones used, the organic phase being the mobile phase, substances having a high R_F value on Systems I, II, and III would be expected to be correspondingly slow moving on System IV. Thus, a decrease in rate of travel is observed for analogous compounds passing from the A series through the B series to the C series. Acetylation of a compound increased its rate of travel in all instances, and addition of a glucose molecule to a desglucoglycoside to produce a deacetyl lanatoside decreased the rate of movement. The main deviation from this rule was found in the behaviour of the genins and the desglucoglycosides. Contrary to expectation the genin, in every case, travelled more slowly than its desglucoglycoside. This indicates that for this system, the hydroxyl group at position C(3) appears to be of greater significance than the hydroxyl groups of the three digitoxose molecules.

The effect of structural differences upon the R_F values so far discussed appears to conform with changes in the polarity of the molecules. However, it is not possible to attribute all changes in R_F values to an increase or decrease in polarity. Oxidation of hydroxyl groups to ketones would be expected to produce a definite lowering of R_F values in the first three systems. Instead it is found that digitoxigenone and digoxigenin-3-one travel ahead of the corresponding genins. Reduction of the unsaturated linkage in the lactone group to give dihydrodigitoxigenin, dihydrodigoxin, and dihydrodigoxigenin, which should result in a decrease in polarity, does not give the expected decrease in R_F values. In these examples it would appear that either partition effects do not follow polarity changes, or that adsorption effects of the paper have an influence on R_F values.

Summary

1. The behaviour of a number of digitalis glycosides and their derivatives has been studied on three chromatographic systems composed of a stationary organic phase and a mobile aqueous phase, and on one system with reversed order of polarities.

2. On the first three systems, members of the C series of digitalis glycosides always travelled ahead, followed by corresponding members of the B and A series in that order. The differences in rate of travel between members of these three series are ascribed to differences in the number and positions of hydroxyl groups on the steroid nucleus.

3. The presence of glucose increases the rate of travel of the glycosides, whereas the presence of digitoxose has little effect. Acetylation of an hydroxyl group retards the movement, whereas conversion to a ketone increases the rate of travel. Formation of the $\Delta^{14:15}$ anhydro compound greatly decreases the rate of travel. Hydrogenation of the lactone ring has little effect.

4. Chromatography of these compounds on the fourth system, as expected, generally reversed the results obtained with the previous systems.

The authors wish to thank Dr. H. Silberman of the Pharmacology Department, University of Sydney, for helpful advice and assistance, Sandoz A.G., Basel, for supplies of lanatosides A, B, and C, deacetyl lanatosides A, B, and C, and acetyldigoxin, and Professor T. Reichstein for *epi*-digitoxigenin.

References

1. Jensen and Tennöe, *J. Pharm. Pharmacol.*, 1955, **7**, 334.
2. Schindler and Reichstein, *Helv. Chim. Acta*, 1951, **34**, 108.
3. Heftmann and Levant, *J. biol. Chem.*, 1952, **194**, 703.
4. Tschesche, Grimmer, and Seehofer, *Chem. Ber.*, 1953, **86**, 1235.
5. Silberman and Thorp, *J. Pharm. Pharmacol.*, 1954, **6**, 546.
6. Silberman and Thorp, *ibid.*, 1953, **5**, 438.
7. Svensen and Jensen, *Pharm. Acta Helvet.*, 1950, **25**, 241.

A NOTE ON THE ACUTE TOXICITY OF HYDROLYSABLE AND CONDENSED TANNINS

By D. M. G. Armstrong, E. G. C. Clarke and E. Cotchin

Departments of Physiology and Pathology, Royal Veterinary College, London, N.W.1.

Received July 4, 1956

In the course of an investigation into the toxicity of the acorn, Clarke and Cotchin[1] found that acorn tannin had a toxicity comparable with that of tannic acid, but were unable to trace experiments on the toxicity of other tannins. Although these substances had long been regarded as the toxic principles of certain plants, little interest had been shown in their toxicity to man until numerous fatalities were reported after the use of tannic acid in the treatment of burns. Cameron, Milton and Allen[2] and others then showed that tannic acid was extremely toxic by injection in laboratory animals, death following severe liver damage. It is important to note, however, that except for some work by Hartman and Romence[3] on quebracho tannin, all these experiments were carried out with tannic acid. This substance, usually obtained from galls growing on the oak (*Quercus* spp.), is but one of many tannins.

Broadly speaking, these substances may be divided into two classes—hydrolysable tannins and condensed tannins. The former on treatment with mineral acids are readily hydrolysed to sugars and gallic acid or ellagic acid, while the latter condense to yield ill-defined insoluble bodies called phlobaphenes. Tannic acid is a member of the first group. Whether the difference in chemical properties between these two groups of tannins was paralleled by difference in toxicity is the subject of this note.

Experimental Procedure

In addition to tannic acid it was decided to use acorn, myrabolam, sumac and chestnut tannins to represent the hydrolysable, and spruce, quebracho, mimosa, mangrove and gambier to represent the condensed tannins. The material was injected parenterally by four different routes in experiments limited to mice.

The tannic acid was of B.P. grade, the acorn tannin was prepared by the method described by Clarke and Cotchin[1] and the other tannins were commercial samples. All solutions were made in distilled water, and were sterilised by autoclaving, which had the additional effect of redissolving any tannin precipitated by dilution. The volume of solution injected was 0·25 ml. Two-fold serial dilution was employed, the largest dose being 1600 mg./kg.

Results

Table I gives the values of the median lethal doses for the various tannins used. They were calculated by Kärber's method[4]. The use of this method was considered to yield values sufficiently accurate for purposes of comparison in view of the wide range of dose used. This

TABLE I

MEDIAN LETHAL DOSES OF CERTAIN TANNINS (Mg./kg.)
KÄRBER'S METHOD

Hydrolysable tannins	Intravenous	Intraperitoneal	Intramuscular	Subcutaneous
Acorn	150 (8)	100 (8)	75 (8)	100 (8)
Myrabolam	150 (9)	150 (8)	150 (8)	150 (8)
Sumac	150 (8)	150 (8)	150 (8)	150 (8)
Valonia	50 (16)	110 (38)	280 (20)	170 (20)
Chestnut	50 (22)	150 (22)	120 (20)	140 (20)
Tannic acid	75 (22)	50 (22)	350 (20)	240 (20)
Condensed tannins				
Spruce	400 (30)	800 (26)	>1600 (30)	>1600 (30)
Quebracho	130 (17)	360 (12)	>1600 (10)	>1600 (10)
Mimosa	130 (45)	320 (32)	>1600 (30)	>1600 (30)
Mangrove	100 (8)	400 (12)	>1600 (10)	>1600 (10)
Gambier	370 (37)	950 (24)	>1600 (24)	>1600 (24)

The figure in parenthesis after each dose indicates the number of mice used in the determination of that dose.

Table, however, does not adequately express the toxicity of these tannins, as it leaves out of account the time factor. This is shown more clearly in Table II, which gives the number of instantaneous and delayed deaths, and the number of survivals, in groups of 20 mice injected with decreasing amounts of various tannins, four mice being injected with each dose. The instantaneous deaths occurred within a few seconds, while the delayed deaths usually took place between the first and the fifth day. Animals that appeared normal on the seventh day were counted as survivors.

TABLE II

TOXICITY OF CERTAIN TANNINS FOR MICE

	Total number of mice injected	Instantaneous death	Delayed death	Survivors
Intravenous:				
Tannic acid	20	16	4	0
Valonia	20	12	8	0
Chestnut	20	16	4	0
Spruce	20	9	3	8
Mimosa	20	15	1	4
Gambier	20	9	0	11
Intraperitoneal:				
Tannic acid	20	0	20	0
Valonia	20	0	18	2
Chestnut	20	0	18	2
Spruce	20	0	6	14
Mimosa	20	0	10	10
Gambier	20	0	5	15
Intramuscular:				
Tannic acid	20	0	13	7
Valonia	20	0	12	8
Chestnut	20	0	17	3
Spruce	20	0	1	19
Mimosa	20	0	4	16
Gambier	20	0	0	20
Subcutaneous:				
Tannic acid	20	0	13	7
Valonia	20	0	15	5
Chestnut	20	0	16	4
Spruce	20	0	0	20
Mimosa	20	0	1	19
Gambier	20	0	2	18

Discussion

With intravenous injection, there is little difference in the lethal dose between the two groups of tannins. For the intraperitoneal route, however, the lethal dose is clearly smaller for the hydrolysable tannins. For the intramuscular and subcutaneous routes the difference between the groups is very marked; in fact with some of the condensed tannins all mice survived the largest dose used (1600 mg./kg.).

Intravenous injection not only failed to show the difference in lethal dose that was shown by injection by other routes, but also appeared to kill in a different fashion. Most of the mice died more or less instantaneously, apparently from embolism due to the precipitating action of the tannins on the blood, and not from any systemic toxic action.

After subcutaneous and intramuscular injection, tannins of both groups caused local necrotic and inflammatory lesions, but histological examination of a limited number of mice suggested that only the hydrolysable tannins produced centrolobular liver necrosis of the kind described by Cameron[2] and others in experiments with tannic acid. This suggests that the condensed tannins are held in combination with protein at the site of injection or at any rate do not diffuse away quickly enough to reach the liver in sufficient quantity to cause necrosis. In this connection Gustavson[5,6] has shown that the condensed tannin from mimosa combines with protein at physiological pH to a much greater extent than does tannic acid, which scarcely combines at all.

On intraperitoneal injection the hydrolysable tannins produced centrolobular necrosis of the liver but there was no clear evidence that the condensed tannins did so. The cause of death in the latter case thus remains undetermined.

These results were obtained in mice. It should, however, be noted that, with dogs, Hartman and Romence[3] found centrolobular necrosis of the liver in two out of four animals after the subcutaneous injection of quebracho tannin.

It seems, then, that one may conclude that the condensed tannins when injected subcutaneously or intramuscularly are much less toxic than the hydrolysable. If this were found to hold for tannins applied to a burned surface, there might be a case for investigating the use of condensed tannins in the treatment of burns.

Summary

1. Differences in chemical properties known to exist between the hydrolysable and the condensed tannins were found to be paralleled by differences in toxicity.

2. Condensed tannins were much less toxic by intramuscular or subcutaneous injection than were hydrolysable tannins such as tannic acid.

3. It is suggested that the use of condensed tannins in the treatment of burns merits experimental investigation.

Our thanks are due to the Calder and Mersey Extract Co. Ltd., Dr. T. White and the Forestal Land, Timber and Railways Co. Ltd., Dr. K. H.

Gustavson, Richard Hodgson and Sons Ltd., and the Walker Extract and Chemical Co. Ltd., for gifts of tannins.

References

1. Clarke and Cotchin, *Brit. Vet. J.*, 1956, **112**, 135.
2. Cameron, Milton and Allen, *Lancet*, 1943, **2**, 179.
3. Hartman and Romence, *Ann. Surg.*, 1943, **118**, 402.
4. Irwin and Cheeseman, *J. Hygiene*, 1939, **39**, 574.
5. Gustavson, *Faraday Soc. Discussions*, 1954, **16**, 45.
6. Gustavson, *J. Polymer Sci.*, 1954, **12**, 317.

INTERFERENCE BY β-METHYL-β-ETHYL GLUTARIMIDE IN THE DETERMINATION OF BARBITURATES

By A. S. Curry

Home Office Forensic Science Laboratory Harrogate

Received August 17, 1956

A rapid and reliable colorimetric method for the estimation of barbiturates is of importance to the clinical biochemist and the forensic chemist. The cobalt acetate-*iso*propylamine reaction was the main method used until recently when the widespread availability of ultra-violet spectrophotometers enabled measurements in the ultra-violet to form the basis of many methods. There are few compounds that interfere in practice with either of these methods[1] but it is the purpose of this paper to show that bemegride (β-methyl-β-ethyl glutarimide), which has recently been introduced as a treatment for acute barbiturate poisoning, can interfere in the quantitative estimation of barbiturates.

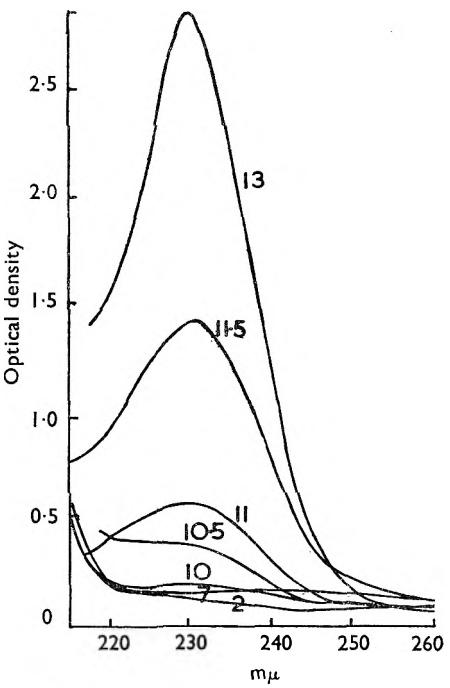


FIG. 1. β-Methyl-β-ethyl glutarimide 2 mg./100 ml. The figures are pH values; that of 11·5 is approximate and represents 0·5N ammonium hydroxide.

Recently in this laboratory it was observed that the absorption maximum of a blood-extract for barbiturate was to be found at 235 mμ in 0·5N ammonia solution instead of at 240 mμ. This observation led to the discovery that bemegride can interfere with the determination of barbiturates by measurements in the ultra-violet. Initial experiments with this compound showed that it was extracted by ether from aqueous acidic solution, that it was not extracted from ether by aqueous sodium bicarbonate solution, and that it was extracted from ether by 0·1N sodium hydroxide. It was thus being extracted together with the barbiturates. A positive cobalt-amine reaction was obtained and it appears that a false result is possible should this method be used to assay barbiturates in the presence of bemegride.

The 5 : 5-disubstituted barbiturates have absorption maxima at 255 mμ in 0·5N sodium hydroxide; this peak increases in intensity and moves to

240 mμ as the pH is decreased to 11·5. Between 11·5 and 9 the absorption is substantially unchanged but with further decrease of pH the absorption decreases until in acid solution it is negligible compared with its value at pH 9 to pH 11·5. The difference in absorption at pH 10–11 and pH 2 forms the basis of most ultra-violet methods of assay. Authors differ on the exact higher pH; McCallum[2] uses 0·5N ammonia solution, Walker and his colleagues adjust the pH of a sodium hydroxide solution to pH 10 with micro drops of sulphuric acid[3], while most other authors use borate buffer at approximately pH 10. Any compound whose absorption at 240 mμ decreases as the pH is decreased from pH 11 to pH 2 can interfere with this method. The ultra-violet spectrum of bemegride and its change with pH is shown in Figure 1.

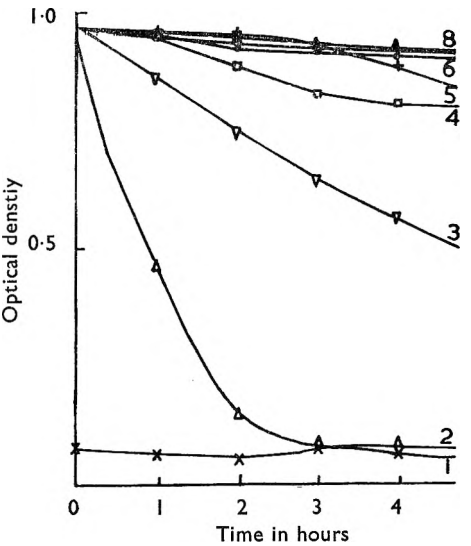


FIG. 2. Hydrolysis rate of bemegride and several barbiturates.

1. Bemegride in acid solution.
2. „ „ 0·5N NaOH.
3. Allobarbitone.
4. Phenobarbitone.
5. Amylobarbitone.
6. Butobarbitone.
7. (Between 6 and 8) Cyclobarbitone.
8. Quinalbarbitone.

It is apparent that, if present, bemegride can interfere with the assay of barbiturates. The Figure also shows that the variation of the spectrum of bemegride with pH is significantly different from that of the barbiturates. At pH 10 and pH 11·5 the absorption of the barbiturates is substantially the same but between these pHs the spectrum of bemegride undergoes radical changes. If, therefore, aliquots of an extract in equal volumes of 0·5N ammonia and borate buffer at pH 10 differ in their absorption at 240 mμ there is presumptive evidence for the presence of bemegride. If the whole spectra are then plotted at pH 9, pH 11·5 and pH 13 the resulting curves will enable an estimate to be made of the contribution of bemegride to the absorption of the barbiturate.

At the present time little is known of the distribution and nature of metabolites of bemegride. A urinary metabolite has been reported[4] and it appears that this compound may also interfere with the determination of barbiturates. A second method of estimating the barbiturate concentration seemed desirable and experiments showed that one method of some value was the hydrolysis of an aliquot under alkaline conditions. The glutarimide ring is apparently less stable than the barbiturate ring and consequently bemegride can be hydrolysed by

conditions affecting only a small proportion of most barbiturates. The hydrolysis products have negligible absorption at 240 mμ in acid and alkaline solution and the barbiturate may thus be assayed as usual after the hydrolysis. The suggested conditions are heating at 38° for 2 hours in 0·5N sodium hydroxide solution. Figure 2 shows the hydrolysis rates of bemegride and several barbiturates under these conditions. The concentrations of the glutarimide were obtained from readings at 230 mμ, while the barbiturate concentrations were followed from readings at 255 mμ.

Summary

1. Bemegride has been shown to interfere with the quantitative estimation of barbiturates.
2. A method which surmounts this difficulty is suggested.

Thanks are accorded to Mr. E. Rutter for technical assistance, to Dr. F. G. Tryhorn for much encouragement, and to Nicholas Products Ltd., for a gift of bemegride.

References

1. Broughton, *Biochem. J.*, 1956, **63**, 207
2. McCallum, *J. Pharm. Pharmacol.*, 1954, **6**, 733.
3. Walker, Fisher and McHugh, *Amer. J. Clin. Path.*, 1948, **18**, 451.
4. McCallum, *J. Pharm. Pharmacol.*, 1955, **7**, 276.

SALINE SOLUTION AS A FACTOR AFFECTING THE TOXICITY OF INTRAVENOUSLY INJECTED TETRACYCLINES IN MICE

By G. Maffii, F. Semenza and E. Soncin

From the Laboratory of Pharmacology, Lepetit S.p.A. 8–10, via R. Lepetit—Milan

Received July 23, 1956

The toxicity of tetracyclines is generally tested by intravenously injecting five mice of uniform weight with 0·5 ml. of a solution containing 2 mg./ml. of the antibiotic under test, in the form of the hydrochloride, over a period of not more than five seconds.

The regulations laid down by the Food and Drug Administration[1] and almost universally accepted, state that the solution of the antibiotics (chlortetracycline and tetracycline) must be prepared in sterile, distilled water, and the mortality must be observed during the 48 hours following injection. These regulations are common to the toxicity tests for other antibiotics: penicillin (alone or combined with vasoconstrictors), streptomycin (alone or combined with penicillin) and dihydrostreptomycin. The use of sterile physiological salt solution is instead prescribed for procaine penicillin, dibenzylethylenediamine-penicillin, penicillin V, chloramphenicol and bacitracin.

The United States Pharmacopeia, XV, does not greatly change the Food and Drug Administration regulations as far as the safety tests for antibiotics are concerned. A solution of sodium aminoacetate must be used for oxytetracycline.

In contrast, the British Pharmacopoeia, 1953, in the test for undue toxicity of chlortetracycline, directs that the solution of the antibiotic may be made either in water or in saline solution. However, it does not specify the rate of injection. In the 1955 Addendum these regulations are not modified.

We would like to draw attention to the importance of using either one or the other solvent in determining the acute toxicity of tetracyclines in mice, by intravenous administration. In fact, we have observed large differences in the mortality rate of animals injected intravenously with tetracyclines, whether distilled water or physiological solution is used as solvent. No record has been found in the literature but Dr. Grove told one of us that he obtained similar results with chlortetracycline.

Materials and Method

CF–1 mice and CF Wistar rats were used for the experiments. Both were bred in our laboratories.

Unless otherwise described, each mouse was injected with 0·5 ml. of solution per 20 g. body weight, the concentration of the drugs being suitably varied, in order to inject the same volume of liquid for each dose. The rate of injection was always 0·1 ml./sec. All injections were by one experimenter and the time was controlled with the aid of a metronome.

Four hours before the test, food was withdrawn and, after injection, the animals were placed, in groups of five, in cages kept in well-ventilated surroundings at a temperature of 23° (±2°). Observations were made for 72 hours. Toxicity tests on the same substance, using different solvents, were carried out on the same day or, at the latest, after 24 hours. Oxytetracycline, chlortetracycline, tetracycline and bromtetracycline were used in the form of the hydrochlorides, and streptomycin as the sulphate. The distilled water and the saline solutions were sterile and pyrogen-free.

The LD50 and confidence limits 19/20 were calculated according to Lichtfield and Wilcoxon[2].

The technique for the *in vitro* and *in vivo* haemolysis tests are described later on.

Results

The intravenous LD50 of tetracycline, oxytetracycline, chlortetracycline, bromtetracycline and streptomycin was determined in mice, using both water and physiological salt solution. As seen in Table I, the values of the LD50 differ significantly when a tetracycline is administered in aqueous instead of saline solution.

TABLE I

LD50 OF THE FOUR TETRACYCLINE HYDROCHLORIDES AND OF STREPTOMYCIN SULPHATE, INTRAVENOUSLY INJECTED TO MICE EITHER IN AQUEOUS OR IN SALINE SOLUTION

Drug	Solvent	Doses	Animals/ dose	LD50 mg./kg.	C.L.*	T.R.†	C.L.‡	Significance of difference (P = 0·05)
Tetracycline HCl (sample 57)	Water	4	20	149	137–162	1·31	1·21–1·41	+
Tetracycline HCl	NaCl 9 g/l.	4	20	195	186–204			
Tetracycline HCl (standard)	Water	4	25	157	144–172	1·42	1·30–1·55	+
Tetracycline HCl	NaCl 9 g/l.	4	25	223	211–236			
Oxytetracycline HCl	Water	6	20	124	114–134	1·43	1·32–1·54	+
Oxytetracycline HCl	NaCl 9 g/l.	4	20	177	169–185			
Chlortetracycline HCl	Water	4	20	101	94–110	1·21	1·07–1·32	+
Chlortetracycline HCl	NaCl 9 g/l.	4	20	122	113–132			
Bromtetracycline HCl	Water	4	20	83	72–95	1·44	1·20–1·72	+
Bromtetracycline HCl	NaCl 9 g/l.	4	20	120	106–136			
Streptomycin H_2SO_4	Water	3	20	120	107–134	1·13	0·98–1·30	–
Streptomycin H_2SO_4	NaCl 9 g/l.	4	20	106	96–117			

* C.L. = Confidence Limits (P = 0·05) of LD50. † T.R. = Toxicity ratio.
‡ C.L. = Confidence Limits (P = 0·05) of the T.R.

The value of LD50 of the same sample of tetracycline dissolved in different concentrations of NaCl (1, 3, 5, 7, and 9 g./l.) again showed wide differences (Table II, Fig. 1). These results strongly suggest a

relation between the concentrations of NaCl in the solvent and the values of the LD50.

The difference in the acute toxicity of tetracyclines in mice was also observed for other species of animals. On intravenously injecting into rats equal volumes of tetracycline solution, corresponding to 2 ml./200 g. of body weight, we obtained a significant difference of the LD50, according to the solvent used. These differences tend to decrease when the volume injected is reduced. As can be seen from Table III, using a volume of 1 ml./200 g. of body weight, the differences are no longer significant.

TABLE II

LD50 OF TETRACYCLINE HCl IN SALINE SOLUTIONS AT VARYING CONCENTRATIONS

Solvent g./l.	LD50 mg./kg. of tetracycline and Confidence Limits
Water	112 [96–130]
NaCl 1	142 [133–152]
,, 3	155 [138–174]
,, 5	172 [155–191]
,, 7	195 [164–232]
,, 9	210 [189–233]

Two groups of five guinea pigs—male and of the same weight (450 g.)—were used to determine the lethal dose of tetracycline, by perfusion into the jugular vein. A constant rate perfusion apparatus was used (3·06 ml./minute). The time of death was checked by electrocardiographic recording. The rate of perfusion and the concentration of the tetracycline solutions were chosen in such a way that the deaths occurred after a reasonably long interval.

TABLE III

DIFFERENT LD50 VALUES OF TETRACYCLINE HYDROCHLORIDE INTRAVENOUSLY ADMINISTERED INTO RATS, ACCORDING TO THE VOLUME INJECTED

Substance	Solvent	ml./200 g. rat	Doses	Animals/ dose	LD50 mg./kg.	C.L.*	T.R.†	C.L.‡	Significance of difference (P = 0·05)
Tetracycline HCl (sample 57)	Water	1	4	10	130	84–202	1·14	0·74–1·77	–
Tetracycline HCl	NaCl 9 g./l.	1	4	10	149	139–160			
Tetracycline HCl (sample 57)	Water	2	4	10	120	107–134	1·46	1·27–1·68	+
Tetracycline HCl	NaCl 9 g./l.	2	4	10	175	160–191			

* C.L. = Confidence Limits (P = 0·05) of LD50. † T.R. = Toxicity ratio.
‡ C.L. = Confidence Limits (P = 0·05) of the T.R.

The results of these experiments (Table IV) confirmed that even in both these species of experimental animals, and under different experimental conditions, the LD50 showed significant changes according to the selected solvent.

TABLE IV

MEDIUM LETHAL DOSES OF TETRACYCLINE HCl BY PERFUSION INTO THE JUGULAR VEIN OF GUINEA PIGS

Solvent	Concentration and pH	Infusion rate	Animals	Median lethal doses mg./kg. ± S.D.
Water	3·5 g./l.–3·2	3·06 ml./min.	5	259 ± 24·8
NaCl 9 g./l.	3·5 g./l.–3·2	3·06 ml./min.	5	449 ± 38·5

Further proof of the importance of the hypotonicity of solutions in affecting tetracycline LD50 values, was obtained by administering to groups of mice, tetracycline dissolved in water, at concentrations higher than in above-mentioned tests.

Using solutions which allowed injection of doses near the LD50 in a volume of solution five times smaller (0·1 ml., instead of 0·5 ml./animal), we found that there was a marked difference in the mortality of the animals treated (Table V). The solubility of tetracycline did not allow the determination of the LD50 using high concentrations, but the differences in mortality for the single doses were undoubtedly significant. In fact, using concentrated solutions, mortality was constantly less than that resulting from corresponding doses at the usual concentration. This fact was not observed when tetracycline dissolved in physiological salt solution was used.

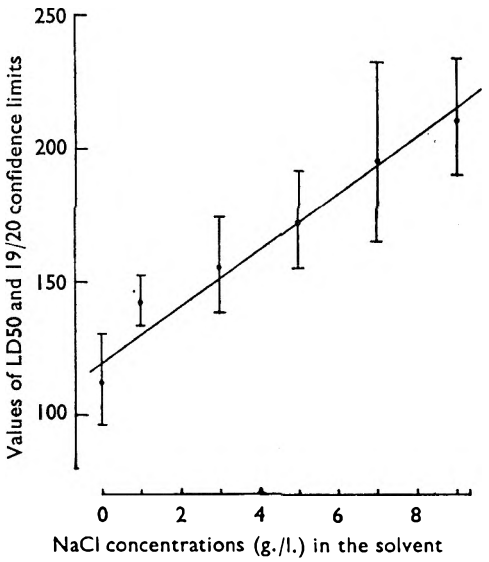


FIG. 1. LD50 values for mice given tetracycline-hydrochloride i.v. in saline solutions of different concentration.

These results show, as could logically be expected, that NaCl has no influence in reducing the immediate mortality from intravenously injected tetracyclines, as the same effect can be obtained by increasing the concentration of the antibiotics in the solutions. On the other hand, it appears that the volume of solution injected has some influence, independent of the molecular concentration.

On the assumption that distilled water may influence the pattern of toxicity produced by intravenously injected tetracyclines, the mechanism of the toxic action could be logically explained by the modifications that hypotonic solutions may produce in the homeostasis of the circulating blood. This effect would be always noticeable when water is used as a solvent for substances which cause alterations of the haematic constants.

We therefore studied the acute toxicity of saponin which has a well-known haemolytic action, and of hydrochloric acid solution, under experimental conditions analogous to those described above. The acute toxicity of these substances was greater when they were administered in aqueous solution. The LD50 values for saponin, intravenously injected into mice, when calculated on the basis of the mortality observed during the 72 hours after treatment, are not significantly different for the two solvents used. If, however, the immediate mortality is considered (within

TABLE V

VARIATIONS OF THE MORTALITY BY TETRACYCLINE HCl ACCORDING TO THE CONCENTRATION OF THE SOLUTIONS INTRAVENOUSLY INJECTED INTO MICE

Drug	Dose mg./kg.	Solvent	Volume injected: 0·5 ml./mouse					Volume injected: 0·1 ml./mouse				
			Per cent concentration	pH	M/1	Treated animals dead	Per cent mortality	Per cent concentration	pH	M/1	Treated animals dead	Per cent mortality
Tetracycline HCl	110	Water	0·44	3·3	0·009	13/20	65	2·2	2·9	0·046	7/20	35
Tetracycline HCl	130	Water	0·52	2·8	0·011	5/10	50	2·6	2·2	0·054	2/10	20
Tetracycline HCl (sample 57)	140	Water	0·56	3·2	0·012	7/20	35	2·8	2·6	0·058	0/20	0
Tetracycline HCl	180	Water	0·72	3·1	0·015	15/20	75					
Tetracycline HCl	250	Water						4·0	2·4	0·083	8/20	40
Tetracycline HCl	110	NaCl 9 g./l.	0·44	3·3	0·162	1/20	5	2·2	2·9	0·199	5/20	25
Tetracycline HCl	130	NaCl 9 g./l.	0·52	2·8	0·164	1/10	10	2·6	2·2	0·207	3/10	30

two hours from injection), it is seen that there are no deaths among the animals treated with saponin in a salt vehicle, while in those treated with aqueous solution mortality was sufficiently high to allow determination of the LD50 (Table VI).

We did not study this interesting experimental fact further, as we felt that it was already possible to establish an analogy between the phenomenon described for the tetracyclines and the results of the immediate

TABLE VI

EFFECTS OF TIME ON THE TOXICITY OF SAPONIN* DISSOLVED IN DISTILLED WATER AND IN NORMAL SALINE

Drug	Solvent	Doses	Animals/ dose	LD50 mg./kg. and Confidence Limits	
				2 hours	72 hours
Saponinum	Water	7	20	27 (18–41)	8·6 (7·2–10·2)
Saponinum	Normal saline	5	25	>50	8·0 (6·1–9·6)

* Saponinum–Merck.

toxicity from saponin, particularly in view of the fact that toxic doses of tetracyclines cause 70 per cent of the deaths within the first 20 minutes after injection.

We then injected intravenously, as above, a toxic dose of hydrochloric acid in distilled water (0·5 ml. of N/4 solution) to two groups of mice weighing 20 g. each. The same dose was repeated, using saline solution and checking potentiometrically the pH which had to be identical in both cases. We found that 13 out of 20 mice (65 per cent) died after 72 hours, when we used aqueous acid solution, while only three out of 20 (15 per cent) died when the same solution contained NaCl.

We feel that these results are sufficiently significant to allow the conclusion that, even in the case of hydrochloric acid solution, the presence of distilled water, i.e., the hypotonicity of the solution, unfavourably influences the resistances of mice to intravenous injection of acid solutions.

As the injection of distilled water alone in volumes equal to those used for tetracycline, did not cause any evident disturbances in mice, the mechanism of action by which distilled water increased in toxicity of these substances was still to be ascertained.

In vitro blood red cell resistance tests were made with rabbit red cells washed and suspended in different concentrations of NaCl, ranging from 0·9 to 0·3 per cent. For adjustment and maintenance of a neutral pH, a phosphate buffer was used. The effective molecular concentration was calculated after the buffer had been added.

No distinct change in the cell resistance in the presence of tetracycline was observed up to concentrations of 416 μg./ml. However, we observed that when control of the red cells suspensions in progressively increasing hypotonic solutions was continued for 16 and 24 hours, at room temperature, there was a decrease of globular resistance in the tubes containing tetracyclines, whilst the values of the maximum haemolytic concentration remained almost constant in the controls.

This point is of limited importance, as in these studies interest was centered on the immediate effect of the tetracyclines. However, it might perhaps indicate a tendency to affect the haematic crasis.

We also examined the behaviour of mice red cells in hypotonic solutions *in vitro*, after treating the animals with tetracycline, but found no difference in the globular resistance compared with the untreated controls.

However, during these tests, we observed that, independently of the globular resistance of the whole cells, the serum of animals treated with toxic doses of tetracycline showed a certain degree of directly observable spontaneous haemolysis. In order to confirm this, we injected toxic doses of tetracycline in physiological saline solution and in distilled water into groups of mice. The tetracycline dose was 190 mg./kg., and the concentration and volume of the solution injected were those normally used in these experiments.

The animals were killed exactly one minute after the end of the injection. After rapid opening of the abdomen and evisceration, 0·1 ml. of blood was drawn out of the abdominal aorta, immediately diluted with 3 ml. of physiological solution and centrifuged for 10 minutes at 1000 r.p.m. After centrifuging it was observed whether the supernatant fluid contained dissolved haemoglobin.

We noticed that in all of the 11 animals treated with tetracycline dissolved in water, the blood showed intense haemolysis, whilst this was seen only in three out of 11 animals treated with tetracycline dissolved in physiological salt solution. We considered the direct observation sufficient, without further determining quantitatively the haemoglobin present.

These tests, although rough and ready, indicate that the presence of distilled water is not without importance, having an influence on the directly observable phenomenon of the lysis of mouse red cells. We do not feel that the phenomenon of haemolysis and that of death must be, in this case, directly connected. In normal conditions, i.e., when

distilled water alone is intravenously injected, there is also a haemolytic effect without death of the animal. However, this tangible aspect of the action of hypotonic solutions may play a part as an accessory factor in causing death in acute intravenous toxicity tests.

Discussion

It is logical to believe that the intravenous administration in hypotonic solution of a given substance, by itself capable of altering the haematic homeostasis, increases the toxic effect and the death rate of the treated animals.

Distilled water alone is not capable of causing death at the doses considered (0·5 ml. per 20 g. of mouse). It also does not affect the mortality produced by toxic doses of drugs which (owing to their molecular weight and the doses at which they may be usefully employed in toxicity tests) do not cause hypotonic conditions or (owing to their pharmacological properties) have no tendency to alter the haematic balance. The rational interpretation of the experimental results suggests some practical conclusions. It is not justifiable to deduce that a given substance whose mechanism of immediate toxicity is unknown, must show, when rapidly injected intravenously into a small animal using a considerable volume of solvent, the same toxicity in water or saline solution. Although it is not excluded that the two data may sometimes be identical, this possibility must still be experimentally tested.

For practical purposes, it is thus essential to specify the solvent to be used in the various "safety" and "toxicity" tests of different official publications, as it is not logical to leave the choice of distilled water or saline solution to the experimenter.

As regards the control tests on tetracyclines, our results have led us to believe that, by using aqueous solutions, the toxic phenomena possibly occurring are not completely attributable to these drugs. In fact it has to be considered that, also at different and lower doses, there is reproduction of those experimental conditions which we have shown to be the least suitable for determining the toxicity of tetracyclines.

In our opinion, it would be more correct to increase the doses of tetracyclines to be used in the "safety" tests, and to employ as a solvent NaCl solution instead of distilled water, although the safety-margin existing with the doses laid down by the U.S.P. and the B.P. exclude a possible interference of the potentiating effects of hypotonic solutions on the toxicity, as described by us.

Summary

1. Tetracycline, chlortetracycline, oxytetracycline and bromtetracycline all show, on intravenous administration to mice, less toxicity when dissolved in saline solution than in distilled water.

2. LD50 values have been determined using both water and saline solution to dissolve the antibiotics. They differ significantly according to the solvent used.

3. Analogous results have been obtained with different species of animals. It is concluded that this phenomenon is directly dependent on the experimental conditions used in performing toxicity tests of tetracyclines and other antibiotic substances.

References

1. *Compilation of Regulations for Tests and Methods of Assay and Certification of Antibiotic and Antibiotic-containing Drugs.* U.S. Department of Health, Education and Welfare, Food and Drug Administration, Vol. I.
2. Litchfield and Wilcoxon, *J. Pharmacol.*, 1949, **96**, 99.

THE OCCURRENCE OF MENTHOFURAN IN OIL OF PEPPERMINT*

By J. A. J. M. Lemli

From the Pharmacognosy Laboratory, the University of Groningen, Holland

Received October 24, 1955

It is generally believed that menthofuran is formed exclusively in the floral organs of peppermint (*Mentha piperita* L.), and it has been detected in oils obtained by distillation of entire flowering plants from different origins (Italy, America, France, Russia, Holland)[1,2].

We have already shown that menthofuran is present in other parts of the plant as well as in the flowers; thus, for example, the very young plants and the purple stolons are rich in menthofuran[1,3]. An abnormally high content has also been found in oil of "basilic mint"[4]. Up to the present time no other evidence for the presence of menthofuran in oils from the foliaceous parts of the plant has been presented, although from a physiological point of view it would be of greatest interest to know if this substance is also secreted in the glandular hairs of the leaves.

Experimental

In order to demonstrate the presence of menthofuran in leaf oils we have employed a colour reaction and also the infra-red absorption curve of this substance. The colour reaction with trichloroacetic acid[5] may also be employed for quantitative estimations[3]. To show the presence of menthofuran in the glandular hairs of a leaf the following reagent was used: lactic acid 40, chloral hydrate 40, trichloroacetic acid 10. The mixture renders the leaves transparent and at the same time colours red the hairs containing menthofuran.

In Figure 1 the secretory glands with high content of menthofuran are more distinct than are those with a low content. It may also be noted that the unicellular glandular hairs are devoid of menthofuran. We may thus conclude that menthofuran is not secreted at the same time in all the glandular hairs. This is to be expected, for the production of essential oil does not commence at the same time in all the glandular hairs on a young leaf. We have already shown that 40 to 60 per cent of glandular hairs are free from oil in a leaf one to two weeks old[6].

The absence of menthofuran from some secretory hairs may also be explained by the fact that when the substance is secreted it is oxidised during the development of the leaf. Menthofuran is rapidly converted into the hydroxylactone which gives no reaction with trichloroacetic acid[6]. Naves[2] found this hydroxylactone in oil of peppermint in which menthofuran could not be detected spectrophotometrically and he believed that the lactone was derived from menthofuran.

Quantitative estimation of menthofuran by means of its colour reaction

* Read at the Medicinal Plants sub-Section of the London meeting of the Fédération Internationale Pharmaceutique on Thursday, September 22, 1955.

with trichloroacetic acid was rejected by Ohloff[7] because he believed it to be non-specific. Nevertheless it is sufficiently specific for oil of peppermint because there are practically no other substances present therein which react with trichloroacetic acid. This is shown by the following assay: a leaf oil, estimated by the method to contain 4·0 per cent of menthofuran, was exposed in thin layers to atmospheric oxidation for 6 months, after which it was found to contain 0·2 per cent of menthofuran by the same method. This latter figure may be regarded as a minimum value given by oils with trichloroacetic acid.

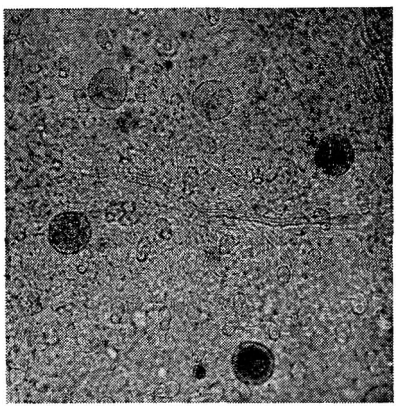


FIG. 1. Glandular hairs on leaf treated with the lactic acid-chloral hydrate-trichloroacetic acid reagent.

Further evidence for the absence of interfering substances is found in the fact that an oil, free from methofuran and giving no reaction with trichloroacetic acid, possesses the same constants as an oil distilled by us and examined by infra-red spectrophotometry.

We have shown conclusively by means of infra-red absorption curves that menthofuran is present in oils distilled from leaves. Naves[2] determined the absorption curve for menthofuran and the curve which we have recorded with the Leitz double beam spectrometer between 1 and 15 μ is reproduced as Figure 2. The absorption bands at 13·1 and 13·6 μ are well suited for the detection of menthofuran since oils free from this substance possess no marked absorption in this region. We have shown that a relation exists between the appearance of these characteristic absorption bands and a positive reaction with trichloroacetic acid. Figure 3 shows the appropriate absorptions between 11 and 15 μ for oils distilled from leaves (*a* and *b*) and from entire plants in full flower (*c*). Menthofuran contents determined by trichloroacetic acid were 0·02, 0·6 and 11 per cent respectively for the three oils. It is seen that a content of 0·02 per cent gives no absorption and that a 0·6 per cent content is already recognisable in the absorption curves. Hence it is concluded that the trichloroacetic acid reaction is sufficiently specific to be used to study the presence of menthofuran in oils of peppermint.

We have used this method of estimation to follow the production of menthofuran during the development of the plant. We believe that the menthofuran contents of oils prepared from entire plants to be of little value for this study because they are a summation of contents of different plant parts of different ages. Thus, in order to obtain a better idea of the production of menthofuran, it is necessary to examine separately oils prepared from leaves of different insertions on the plant and hence belonging to different stages in the vegetative cycle of the plant.

It should also be emphasised that the absolute quantities of menthofuran

in a plant organ must be determined rather than the percentage of it present in each oil; otherwise variations in menthofuran produced by the leaf may be masked by variations in total oil yielded. Thus the total menthofuran produced by each leaf at each insertion has been calculated.

Figure 4 shows the results of these analyses of oils obtained from leaves on the main stem (insertions V, VII, IX and X) and those of the branches ($IV_z + V_z$). Different samples were collected at different stages in the

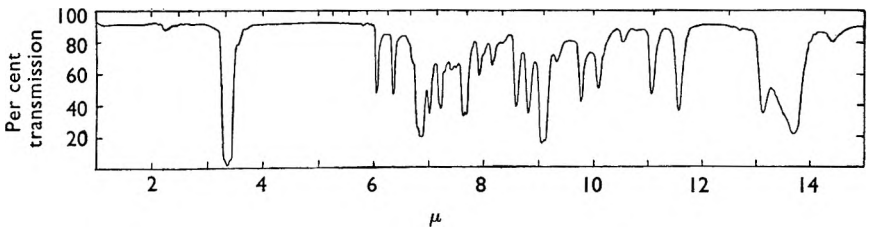


FIG. 2. Infra-red absorption curve of menthofuran.

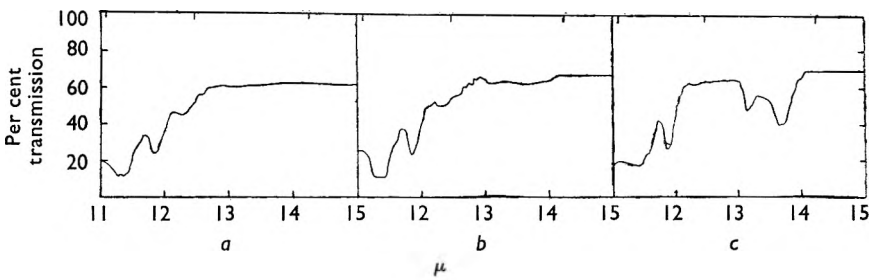


FIG. 3. Infra-red absorption curves of peppermint oils containing: *a*, 0·02; *b*, 0·6 and *c*, 11 per cent menthofuran.

vegetative cycle of the plant; A to E representing stages from young plant to development of flower buds; F to H covers the period of flowering. It is seen that the quantity of menthofuran is a maximum in the young leaves and it diminishes progressively with increased age of the leaf. This is most pronounced in leaves of the tenth insertion where the menthofuran decreases from 23 to 2 μg. For leaves from the branches ($IV_z + V_z$) the curve is more gradual because all the leaves of differing ages from the branch were distilled together.

If we consider the development of menthofuran in the tips of the branches (Table I) we find the converse phenomenon; the absolute

TABLE I

MENTHOFURAN CONTENTS OF OILS FROM BRANCH TIPS
PER CENT

Height of insertion of branches	Period of Collection					
	D	E	F	G	H	J
X	1·3	1·3	1·7	2·6	7·2	20·2
VI	2·0	2·0	2·3	6·3	13·2	—

TABLE II

MENTHOFURAN CONTENTS OF FLOWER OILS

Year	Flower buds		Freshly opened flowers		Full flowers		Fading flowers	
	Oil per cent	Menthofuran per cent	Oil per cent	Menthofuran per cent	Oil per cent	Menthofuran per cent	Oil per cent	Menthofuran per cent
1952	0·392	26·2	—	—	0·412	28·5	—	—
1953	0·480	36·7	0·386	37·2	0·320	36·8	0·289	32·0
1954	—	—	0·495	23·4	0·444	26·6	0·456	20·0

content of menthofuran increases due to the formation of flower buds possessing a very high content of this substance.

The amount of menthofuran in flower buds and flowers is very high but differs in different years of cultivation. After flowering both the oil content and the menthofuran content decreases as shown in Table II.

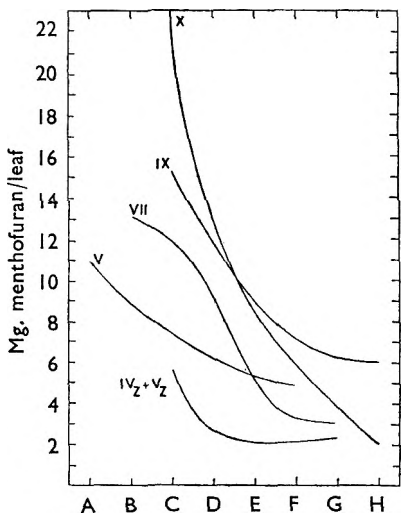


FIG. 4. Mg. of menthofuran present in the leaves of the main stem (insertions V, VII, IX, X) and the branches (IV + V), during the vegetative cycle of the plant (A–H).

Because of the variations outlined above, the percentage of menthofuran present in oils distilled from the entire plants is found to decrease and then to increase as shown in Figure 5. The curves represent the oil content and its menthofuran content as functions of the age of the plant in weeks. The oil of the youngest plants (first week) possesses the highest content of menthofuran. The amount decreases as the plant develops but increases again from the time of formation of flower buds (12th week), finally decreasing once more after flowering (24th week).

From these results it appears that menthofuran is only formed in the young parts of the plant. After a certain age it is secreted no more and that present in the glandular hairs is slowly oxidised to the hydroxylactone by the air. This is also confirmed by the fact that the basal part of a leaf contains a higher percentage of menthofuran than does the distal part, where the glandular hairs are not so young. From these cut leaves we have found the same yields of oils but with menthofuran contents of 1·9 and 0·6 per cent respectively.

DISCUSSION AND CONCLUSIONS

Menthofuran is a substance secreted in the young parts of the plant, that is where metabolism is most active. This explains the high content of the substance in flowers and stolons. The menthofuran content of

basilic mint oil is 20 to 25 per cent and at first sight this is abnormally high; it is, however, of the same order as that from young plants and stolons of healthy plants which contain 12 to 28 per cent.

Such values are quite normal for a young tissue. Now basilic mint is peppermint infected with the parasite *Eriophyes menthae*, family Acaridae, which results in atrophy of the plant, suppression of flowering and the production of abundant terminal masses of numerous, small, appressed leaves. Hence, such diseased basilic shoots are young tissues from the chemical point of view. Thus the composition of oil of basilic mint corresponds to that of young leaves and stolons of typical peppermint[2,6].

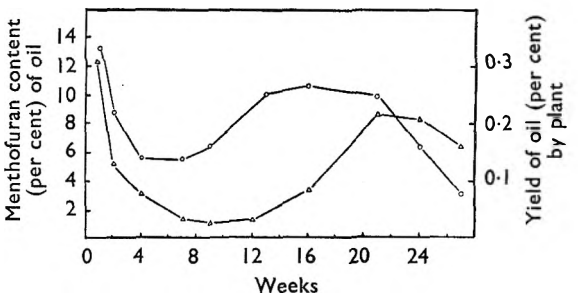


FIG. 5. Oils from entire plants.
○——○ yield of oil (per cent) by plant.
△——△ menthofuran content (per cent) of oil.

Menthofuran is thus shown to be secreted exclusively as a product of young tissues. Since this substance influences the quality of oil of peppermint and since its localisation and formation are understood, it will be possible to employ this information in producing oils of finest quality.

REFERENCES

1. Lemli, *Pharm. Tijdschr. Belg.*, 1955, **32**, 75.
2. Naves, *Bull. soc. chim. Franc.*, 1954, **21**, 657.
3. Lemli, *Ann. pharm. Franc.*, 1954, **12**, 275.
4. Benēzet and Igolen, *Bull. soc. chim. Franc.*, 1951 **18**, 912.
5. Krupski and Fischer, *J. Amer. pharm. Ass. Sci. Ed.*, 1950, **39**, 433.
6. Lemli, *Pharm. Weekbl.*, 1955, **90**, 777.
7. Ohloff, *Arch. Pharm.*, 1952, **285**, 353.

A NOTE ON TRIPELENNAMINE HYDROCHLORIDE AND LOCOMOTOR ACTIVITY

By E. M. Boyd and M. A. Boyd

From the Department of Pharmacology, Queen's University, Kingston, Ontario, Canada

Received August 7, 1956

In 1954, Baird and Boyd reported[1] that tripelennamine hydrochloride had a statistically insignificant effect upon locomotor activity of albino rats measured in Wahmann drums. They found some indication, from the available data, that "locomotor activity of the more active rats might be increased by the smaller doses, and decreased by the larger doses, of tripelennamine hydrochloride", and also that "the locomotor activity of the less active rats might be augmented by the higher doses, and not affected by the lower doses, of tripelennamine hydrochloride". Affirmation of these possibilities could provide evidence that both the sedative and excitant side-reactions of this antihistamine agent are measurable in animals and this work was directed toward that purpose.

One hundred individually identifiable adult male albino rats were acclimatized to the Wahmann drum by the technique previously described[1]. Each rat was starved for 72 hours, placed in the drum for 24 hours, and this repeated at the end of 1 and 2 weeks. The 50 rats which rotated the drum the greatest number of times were separated into the "more active" group and the remaining 50 into the "less active" group. The 50-rat groups were each divided into subgroups of 5 rats. Each of the 5 rats received in rotation at intervals of 1 week, until crossover was complete, subcutaneous injections of tripelennamine hydrochloride in doses of 0, 0·5, 1·0, 2·0, and 5·0 mg. per kg. body weight. These relatively small doses are approximately equivalent to those used therapeutically. The antihistamine agent was dissolved in sterile 0·85 per cent sodium chloride solution and given in all cases in a volume of 1 ml. per kg. weight. The drug was administered after each acclimatized and tested rat had been in the Wahmann drum for 3 hours. Hourly readings on the Veeder counter were recorded for 4 hours after drug administration. From the hourly number of rotations of the drum after the rat had received tripelennamine was subtracted the number of rotations in the corresponding hour after the rat had received a saline injection. These differences were averaged and subjected to a t test of the probability of the mean difference ($\overline{X}_D$ (drug-control)) being zero[2]. The results are summarized in Table I.

At no time were effects obvious to casual observation of the animals. Measurable effects of tripelennamine were confined largely to the second hour after administration. Effects significant at $P \leqq 0{\cdot}05$ were: (*a*) *increased* locomotor activity of the more active rats after receiving a dose of 0·5 mg. per kg. body weight of tripelennamine hydrochloride; (*b*) *decreased* locomotor activity of the more active animals after a dose of

TABLE I

THE INFLUENCE OF TRIPELENNAMINE HYDROCHLORIDE UPON LOCOMOTOR ACTIVITY OF ALBINO RATS

Dose (mg. per kg. subcutaneously)	Change in locomotor activity* per cent hours after drug			
	1	2	3	4
More active animals, N = 49				
0·5	+ 20 (0·2)	+ 55 (0·02)	− 4 (0·8)	+ 11 (0·6)
1·0	− 16 (0·4)	+ 23 (0·3)	− 8 (0·7)	+ 6 (0·8)
2·0	− 14 (0·5)	+ 27 (0·2)	+ 13 (0·5)	+ 5 (0·8)
5·0	− 56 (<0·001)	− 8 (0·7)	+ 1 (0·9)	+ 8 (0·8)
Less active animals, N = 50				
0·5	+ 9 (0·8)	+ 96 (0·2)	− 40 (0·2)	− 10 (0·7)
1·0	+ 24 (0·5)	+ 54 (0·3)	− 11 (0·7)	− 10 (0·7)
2·0	+ 35 (0·3)	+ 154 (0·02)	+ 42 (0·2)	+ 7 (0·8)
5·0	− 24 (0·3)	+ 112 (0·05)	+ 18 (0·6)	+ 76 (0·1)

* $(\bar{X}_D$ (drug-control)/$\bar{X}$ control) × 100 (*P* difference equals zero).

5·0 mg.; (*c*) *increased* locomotor activity of the less active rats following doses of 2·0 and 5·0 mg.

SUMMARY

Measurable sedation and measurable excitation of locomotor activity of albino rats by tripelennamine hydrochloride were demonstrated at selected doses in selected groups of animals by a rotation cross-over assay.

REFERENCES

1. Baird and Boyd, *J. Pharm. Pharmacol.*, 1954, **6**, 398.
2. Croxton, *Elementary Statistics with Applications in Medicine*, New York, Prentice-Hall, Inc., 1953.

RAUWOLFIA ALKALOIDS: FRACTIONATION BY COUNTERCURRENT DISTRIBUTION*

By R. P. Banerjee, M. L. Chatterjee and H. F. Häusler†

From the Department of Pharmacology, School of Tropical Medicine, Calcutta

Received September 11, 1956

Interest in the therapeutic value of rauwolfia has prompted many investigations to find newer active principles therein. A recent review[1], in cataloguing the alkaloids so far reported from different varieties of rauwolfia, lists more than two dozen alkaloids isolated from *R. serpentina* alone though a few of them, it is stated, may be identical. The catalogue may be further extended by including those found in other varieties of rauwolfia, namely *R. canescens*, *R. caffra*, *R. vomitoria*, and *R. heterophylla*.

In isolating and studying the properties of the alkaloids, investigators have used as starting materials drugs collected from widely scattered regions of the world. In the earlier work of Siddiqui and collaborators[2–5], *R. serpentina* roots from Behar were used leading to the isolation of five crystalline alkaloids, namely ajmaline, ajmalinine, ajmalicine, serpentine and serpentinine. Van Italie and Steenhauer[6] used an African variety of the same drug to isolate rauwolfine. Later work on *R. serpentina* Benth has produced many more alkaloids including reserpine[7], sarpagine[8], rauwolfinine[9], reserpinine[10], rauhimbine, and isorauhimbine[11]. Chatterjee and Bose[12] have found serpine in *R. serpentina* roots of the Cochin (S. India) variety where ajmaline is reported to be entirely absent. A strongly active alkaloid, rescinamine, was reported by Klohs and his colleagues[13] in the oleoresin fraction.

In addition to those mentioned above other alkaloids have been isolated or reported to be present in *R. serpentina* from different sources and further additions must be expected.

Two principal preparations have been applied therapeutically: (i) the total alkaloids consisting of an alcoholic percolate of the roots, modified or adjusted to proper alkaloid concentration and (ii) reserpine, alone or in combination with other hypotensive and sedative drugs.

The pharmacology of reserpine has been worked out in great detail by Bein[14], Plummer and others[15], and Gaunt and others[16]. Several workers have studied the pharmacology of other individual alkaloids like ajmaline[17–19], serpentine[20–21], rauwolfinine[22], serpine[23] and rescinamine[24]. Notwithstanding these studies, the pharmacology of the total alkaloidal preparation is far from being completely understood. The "total alkaloids" is a mixture of many components and the effect of administering the total alkaloids is naturally dependent on an interplay of actions

* An enquiry under the Indian Council of Medical Research, dedicated to Sir Henry Dale in honour of his 80th birthday.

† WHO Professor of Pharmacology 1954–55, Head of the Pharmacological Institute, University of Graz, Austria.

of the individual alkaloids some of which incidentally may possess antagonistic properties. An understanding of the pharmacology of the total alkaloids is not easy in view of the difficulty of obtaining the pure alkaloids.

Understanding of the pharmacology of rauwolfia would be hastened if it were possible to know what specific alkaloids occur in a variety of rauwolfia and in what proportions. Not all the catalogued alkaloids would be found in any single variety harvested from a specific locality. The present investigations were undertaken with a view to exploring the possibility of the recently introduced technique of countercurrent distribution to achieve the ends stated above. Experiments described here concern the soluble alkaloids only, and exclude those found in the oleoresin fraction.

Technique

The technique of the countercurrent distribution as developed by Craig and his collaborators has already found many applications in organic and biological chemistry in testing the purity of preparations and particularly in isolating individual components from a mixture of closely related entities. Based on the fundamental postulation of Nernst[25] on the specificity of distribution coefficient for a solute between two immiscible solvents, the method has grown in the last few years into a valuable fractionation tool with wide applicability[26]. Fried and others[27] have employed the technique, using a small scale apparatus, for the fractionation of germidine and germitrine, new alkaloids from *Veratrum viride*. Dorfman and others[28] report about the use of countercurrent extraction in the isolation of reserpine from the oleoresin fraction of *Rauwolfia serpentina*. Some other profitable applications of the technique include the isolation of metabolites of quinine in a pure form[29].

Apparatus

A cylindrical unit consisting of a circular array of stainless steel tubes described by Craig and others[30] has since been discarded in favour of all glass systems. A medium sized glass train of 80 units was designed and built, following essentially the same design for equilibration cell and rocker unit used by Craig in recent work. In trial experiments a mixture of known amino-acids was quantitatively separated by distribution between *n*-butanol and 5 per cent hydrochloric acid[31].

Material and Methods

Ground roots (2 kg.) of *R. serpentina* Benth (Dehra Dun) were exhaustively percolated by 95 per cent ethanol in the cold. The combined percolate was concentrated under reduced pressure and the resulting syrupy mass poured dropwise into a mechanically stirred volume (500 ml.) of 2 per cent hydrochloric acid. The stirring was continued for two hours and the dark brown solution was filtered off. The insoluble material including the resinous mass was again treated with 200 ml. of 2 per cent hydrochloric acid for one hour with constant stirring and filtered. The combined filtrates were made alkaline (pH 11·5) with caustic soda and

completely extracted with chloroform (tested with Meyer's reagent). The deep brown combined chloroform extract was extracted again with 2 per cent hydrochloric acid to remove colour and non-alkaloidal impurities followed by alkalization of acid extract and finally extraction with chloroform.

Preliminary Distribution Studies

An aliquot of the chloroform extract was concentrated to 20 ml. and 10 ml. portions put into the first two tubes of the countercurrent distribution apparatus. After being distributed between chloroform (lower phase) and 0·2M phosphate buffer pH 4·7 (upper phase) using 34 transfers

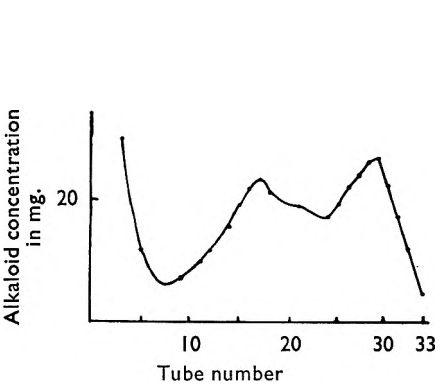


FIG. 1. Showing the alkaloid content (in mg.) of countercurrent tubes plotted against corresponding tube no. Solvents: chloroform and 0·2M phosphate buffer pH 4·7.

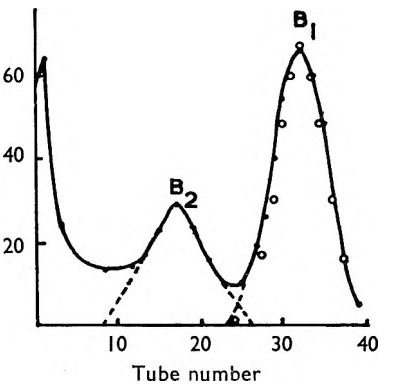


FIG. 2. Showing the countercurrent distribution of tentative fraction A between chloroform and 0·2M phosphate buffer pH 5·6. White circles represent points calculated for ajmaline.

the alkaloid distribution pattern was worked out using gravimetric method of analysis.

The concentration of solute in chloroform phase was determined by directly weighing the residue from 2 ml. aliquots after a wash with dilute ammonia water, using small glass crucibles described by Craig[32].

Alkaloid concentration in the aqueous phase was determined by alkalizing an aliquot, extracting into chloroform, and gravimetric determination of the washed chloroform layer using similar containers for evaporation.

A plot of the alkaloid content against tube number results in a distribution pattern exemplified by Figure 1. Besides yielding expected evidence of heterogenity in the system, this distribution pattern helps to plan future experiments. The material contained in the band represented by tube Nos. 24 to 33, and 8 to 23, for example, can be taken out and subjected to further enquiry using other solvents.

Tentative Fractionation by Extraction with Buffer

Based on the preliminary distribution experiments the following procedure was adopted. A chloroform solution of the total alkaloids

represented by the original chloroform extract is repeatedly extracted in a separating funnel with portions of 0·2M phosphate buffer of pH 4·7 to dissolve a major part of the alkaloids. The combined chloroform extracts are designated tentative fraction A. The chloroform residue is further extracted repeatedly, using a 0·2M phosphate solution having pH 3·0 (prepared by adding 0·2M phophoric acid to 0·2M potassium biphosphate until the resulting pH is 3·0) when further alkaloids are brought into the aqueous phase to make the tentative fraction B. The residue from this step[2] contains other alkaloids and is designated tentative fraction C.

Countercurrent Distribution of Tentative Fractions using Different Buffers

The material in fraction A, transferred to a small volume of chloroform, is again distributed between chloroform and 0·2M phosphate buffer pH 5·6 using 39 transfers. Analysis of the resulting alkaloid distribution pattern (Fig. 2) reveals the presence of two different components. A

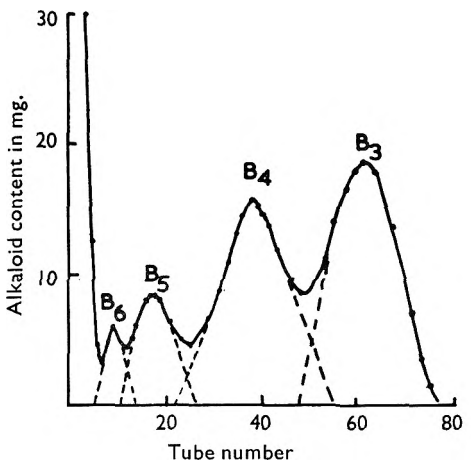


FIG. 3. Showing the countercurrent distribution of tentative fraction B between chloroform and 0·2M phosphate (pH 3·6).

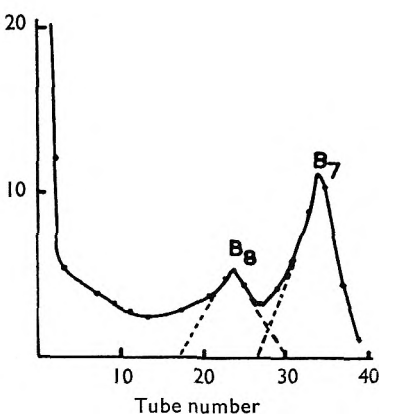


FIG. 4. Showing the distribution of tentative fraction C between chloroform and 0·2M phosphate (pH 1·8).

small amount left over in the first few tubes represents other components having small K-values for buffer (4·7)-chloroform system but carried over in minute quantities. This small amount is therefore mixed with tentative fraction B which by similar manipulation is countercurrently distributed between chloroform and a phosphate solution of pH 3·6 when a pattern represented by Figure 3 is revealed on analysis. The presence of four components is made obvious on examination of the distribution pattern. Countercurrent distribution of tentative fraction C (together with left-over from fraction B) between chloroform and 0·2M phosphate solution of pH 1·8 resolves the fraction into a further two components (Fig. 4). The residue from this operation constitutes a considerable proportion (about 60 per cent) of the starting material (tentative

fraction C). It shows copious precipitation with Meyer's reagent, and is amenable to further fractionation.

Results and Discussion

By operations like the above more than eight clear fractions are obtained from the original material. The fractions may be recovered quantitatively by permitting the operation to continue with a larger number of transfers until the peaks completely disengage from each other.

Identity of Countercurrent Fractions

The identity of the isolated alkaloids may be established chemically or by reference to the known partition ratios where pure alkaloids are available. A knowledge of these constants enables the positions of their maxima in hypothetical countercurrent distribution to be calculated by making use of the equation[26]:

$$N = \frac{n.K.r}{K.r + 1}$$

where N is the position of the maximum,
n = total number of transfers,
K = partition ratio,
r = ratio of volume of upper and lower phases.

Only some rauwolfia alkaloids are available in pure form. A specimen of pure ajmaline which was available gave a value of 4·6 for partition constant between 0·2M phosphate buffer of pH 5·6 and chloroform. Using the above equation the calculated position for maximum corresponds exactly to the experimentally found maximum for the fraction B_1.

An assessment of the purity of one countercurrent fraction could however be made, taking the pure sample of ajmaline as guide. Employing the experimentally determined value of 4·6 for K, the theoretical distribution curve for ajmaline could be calculated using the equation[26]

$$y = \sqrt{(2\pi nK/[K + 1]^2)}\ \exp\left(-\frac{x^2}{2nK/(K + 1)^2}\right)$$

where y = the fraction of substance in a given tube,
K = partition ratio,
n = number of transfers,
x = distance from the maximum of the tube in question.

Since the position of maximum in actual distribution coincided with the position of experimentally determined value of N for ajmaline, for example tube 32, other points in the theoretical distribution pattern for ajmaline are obtained from the above equation by calculation. Figure 2, where the calculated points for ajmaline have been shown together with the experimental curve for fraction B, indicates good agreement. The countercurrent fractions would therefore make excellent starting materials for the preparation of the respective alkaloids in a pure form.

Assay of Preparations

The alkaloid distribution curves may be used to assay the total individual alkaloidal content. A complete separation of the peaks by a large number

of transfers may be dispensed with and a situation represented by Figures 2–4 may suffice. In the estimation of plasma or serum components by electrophoretic techniques, it is often permissible to exercise some judgement for the construction of individual protein peaks by extrapolation in these regions where adjacent peaks might overlap. In the same way the alkaloid distribution patterns obtained in the above experiments may be drawn, but always keeping in mind the requirements for quantitative representation. The experiment depicted in Figure 4 concerned with fractionation of tentative fraction C was, for example, made with material obtained from 0·6 kg. of dried roots in order to have sufficient mass to start with, while 0·3 kg. roots would be found more than adequate for the treatment of tentative fraction A. Where making quantitative interpretation it would of course be necessary to reduce the curves to proper scale as in Figure 5. The proportion of each component in the total preparation is obtained by dividing the area under the corresponding peak by the total area covered, a procedure familiar in quantitative paper electrophoresis. Calculation, in this manner of the percentage of fractions B_1, B_2, etc., gives the following values: B_1 46; B_2 20·5; B_3 10·1; B_4 9·38; B_5 2·74; B_6 1·0; B_7 3·13; B_8 1·61 per cent.

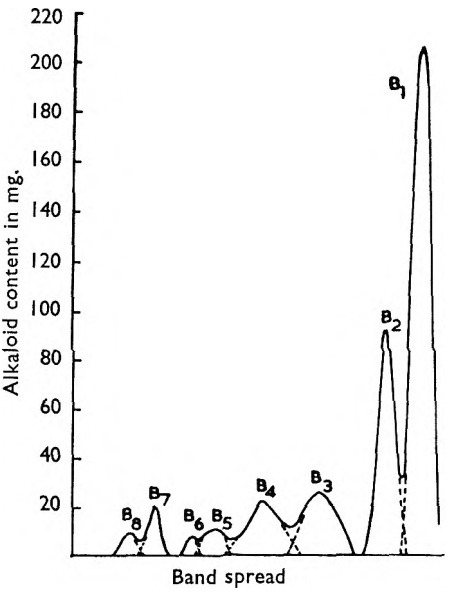


FIG. 5. Showing the quantitative pattern as would result following fractionation by countercurrent distribution of 4090 mg. total alkaloids.

The quantitative assessment of alkaloid distribution in varieties of *Rauwolfia serpentina* harvested from different regions and under different conditions is of interest and economic significance. It is intended here only to point out the possibility of applying the above technique to the problem.

The Character of the Fluorescence in Ultra-violet Light. Preparations of total rauwolfia extracts always show a very strong fluorescence. Some earlier work[33] has been done on the assumption that the fluorescence intensity in dilute solution could be taken to be a measure of the alkaloid concentration and hence measurement of fluorescence could be employed for the estimation of microquantities of alkaloids in urine or other biological materials. It is interesting to find here that the assumption does not hold good for a preparation of total alkaloids. It was considered worth while to follow the fate of the fluorescence as the fractionation of the alkaloid mixture by countercurrent distribution progressed. The

fluorescence intensity of a diluted (1:50) portion of the upper (buffer) phase was measured in a Lumetron Fluorescence meter against a quinine standard (1·2 μg./ml.) and plotted against corresponding tube no. in Figure 6 in which is plotted the relative fluorescence intensity of upper phase against its alkaloid content. The fluorescence though fractionating at the same time, shows maxima in regions not always corresponding to the alkaloid maxima except in two cases, i.e., B_3 and B_7. While it is probable that B_3 and B_7 may be fluorescing alkaloids, it is evident that the rest of the fluorescence distribution throughout the countercurrent train cannot belong to any principal alkaloid and must belong to other material having little relative mass. For example, in the case of B_1, very high intensity of the fluorescence in the upper phase to the right of the alkaloid maximum has not been able to distort the weight distribution curve to any appreciable extent from its symmetrical nature. This holds good for several other regions. These findings call for caution in employing the observation of fluorescence under ultra-violet for the purpose of locating and assigning R_F values to these alkaloids on paperchromatograms as practised by Pillay and others[34].

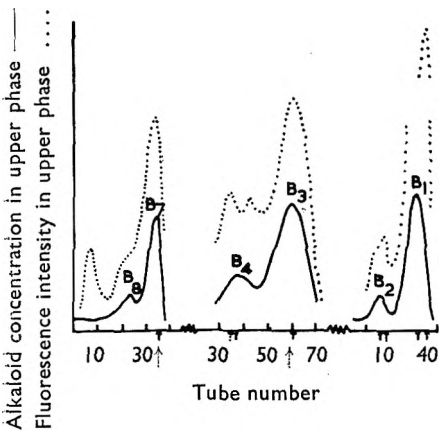


FIG. 6 (not to scale). Where the fluorescence intensity of upper phases (in arbitrary units) has been compared with the alkaloid concentration (in mg.) in the same. The presence of several fluorescence maxima are apparent, but while two of them coincide with the alkaloid concentration maxima for B_3 and B_7, others do not.

SUMMARY

1. A crude preparation of total alkaloids of *Rauwolfia serpentina* Benth yields on countercurrent distribution between chloroform and selected phosphate buffers more than eight fractions containing different components in nearly pure form. These fractions are convenient starting points for the preparation of pure crystalline materials.

2. Countercurrent distribution of total alkaloids in the above manner permits the assay of different preparations.

3. The strong greenish-blue fluorescence usually associated with total extract is composite in nature. While evidence has been obtained for the presence of two fluorescent alkaloids, most of the fluorescence appears to be non-alkaloidal in origin and associated with a molecule of little comparative mass.

4. The pharmacological evaluation of countercurrent fractions may aid in unravelling the intricacies of the pharmacology of total alkaloids.

The authors wish to express thanks to Dr. R. N. Chakravarty and Dr. P. K. Dutta who kindly performed some of the spectrophotometric measurements. Dr. Chakravarty also made available a sample of pure ajmaline for which grateful acknowledgment is made.

REFERENCES

1. Mukherji, *J. Sci. Ind. Res.* (*India*), 1955, **14A**, No. 7 (Supp.), 1.
2. Siddiqui and Siddiqui, *J. Ind. chem. Soc.*, 1931, **8**, 667.
3. Siddiqui and Siddiqui, *ibid.*, 1932, **9**, 539.
4. Siddiqui, *ibid.*, 1935, **12**, 37.
5. Siddiqui, *ibid.*, 1939, **16**, 421.
6. Van Itallie and Steenhauer, *Arch. pharm.*, 1932, **270**, 313.
7. Schlittler and Schwarz, *Helv. Chim. Acta*, 1950, **33**, 1463.
8. Stoll and Hoffmann, *ibid.*, 1953, **36**, 1143.
9. Chatterjee (née Mukherjee) and Bose, *Science and Culture* (*India*), 1951, **17**, 139.
10. Schlittler, Saner and Muller, *Experientia*, 1954, **10**, 133.
11. Hofmann, *Helv. Chim. Acta*, 1954, **37**, 314.
12. Chatterjee and Bose, *Experientia*, 1954, **10**, 246.
13. Klohs, Draper and Keller, *J. Amer. chem. Soc.*, 1954, **76**, 2843.
14. Bein, *Experientia*, 1953, **9**, 107.
15. Plummer, Earl, Schneider, Trapold and Barett, *Ann. N.Y. Acad. Sci.*, 1954, **58**, 8.
16. Gaunt, Renzi, Antonchak, Miller and Gillman, *ibid.*, 1954, **59**, 22.
17. Chopra and Chakravarty, *Ind. J. med. Res.*, 1948, **29**, 763.
18. Raymond Hamet, *C. R. Soc. Biol. Paris*, 1940, **134**, 94, 369.
19. Das Gupta and Werner, *Bull. Calc. School Trop. Med.*, 1954, **1**, (2), 16.
20. Raymond Hamet, *C. R. Acad. Sci. Paris*, 1944, **211**, 414.
21. Das Gupta, Ray and Werner, *Proc. Symp. Indigenous Drugs and Insecticide Nat. Inst. Sci. India*, 1955, Bull. No. 4, 35.
22. Chatterjee and Bose, *ibid.*, 1955, Bull. No. 4, 32.
23. Das Gupta and Werner, *Bull. Calcutta School Trop. Med.*, 1954, **1**, (1), 1.
24. Cronheim, Brown, Cawthorne, Tockes and Ungari, *Proc. Soc. exp. Biol. N.Y.*, 1954, **86**, 120.
25. Nernst, *Zeitschr. Physik. Chem.*, 1891, **8**, 110.
26. Craig, Hausman, Ahrens and Harfenist, *Analyt. Chem.*, 1951, **23**, 1236.
27. Friend, White and Wintersteiner, *J. Amer. chem. Soc.*, 1949, **71**, 3260.
28. Dorfman, Furlenmeier, Huebner, Lucas, MacPhillamy, Mueller, Schlittler, Schwyzer and St. Andre, *Helv. Chim. Acta*, 1954, **37**, 59.
29. Brodie, Baer and Craig, *J. biol. Chem.*, 1951, **188**, 567.
30. Craig and Post, *Analyt. Chem.*, 1949, **21**, 500.
31. Banerjee and Häusler, *Bull. Calcutta School Trop. Med.*, 1955, **3**, 109.
32. Craig, *Methods of Medical Research*, Vol. 5, 1952, p. 16.
33. Gupta, Roy, Ray and Ganguly, *Ind. J. med. Res.*, 1950, **38**, 67.
34. Pillay, Rao and Rao, *Ind. J. Pharm.*, 1955, **17**, 91.

THE COMPARATIVE ANTIPYRETIC ACTIVITY OF ACETYLSALICYLIC ACID AND SALYCYLAMIDE IN FEVER-INDUCED RATS

BY R. H. BULLER, T. S. MIYA* AND C. J. CARR

From the Department of Pharmacology, School of Pharmacy, Purdue University, Lafayette, Indiana

Received September 3, 1956

THE literature reveals relatively few investigations of the antipyretic activity of salicylamide, although many have been conducted on its analgesic activity and ultimate fate. This investigation was initiated to determine the extent of antipyretic activity of salicylamide as compared to that of acetylsalicylic acid and a salicylate combination[1]. The combination (the P-tablet)† contains both the above ingredients in addition to caffeine, aluminium hydroxide, tartaric acid and thiamine hydrochloride. The antipyretic action was also compared with the plasma-salicylamide level. The antipyretic activity of salicylamide has been reported to range from none detectable to equal or greater than aspirin[2–9] in rats, rabbits and man. The fact that salicylamide demonstrates desirable qualities as an analgesic[2,4,6,7,10,11] but inferior qualities as an antipyretic is of interest. The possible cause has not been elucidated, but it is suggested that this drug may be effective as an antipyretic only in the free form, whereas protein-binding and conjugation which ultimately results apparently has no effect on its activity as an analgesic.

EXPERIMENTAL

Experimental Animal. Adult albino Spragus-Dawley rats of mixed sex varying in weight from 200–350 g. were employed. They ate a standard proprietary laboratory diet, with water *ad libitum.* Drugs were administered to pyrexic animals after 12–16 hours fasting.

Fevering Agent. Animals were rendered febrile by subcutaneous injection of 0·6 ml. of a 5 per cent aqueous solution of Witte Peptone. When filtered and incubated for 12–24 hours at 98·6° F., this agent produced fevers averaging 103° F., four hours after injection. Storing under refrigeration preserved its activity indefinitely.

Instruments. A Tele-thermometer was used inserting the rectal electrode approximately two inches. Faecal matter was previously removed by gentle digital pressure and the rectum lubricated with liquid paraffin.

Administration of Drugs. Drugs were suspended in a 0·5 per cent solution of Kelcosol‡ in water and administered orally, four hours after peptone injection, by means of a stomach tube. All concentrations of the

* Present address, Univ. of Nebraska, College of Pharmacy, Dept. of Pharmacology Lincoln 8, Nebraska.

† An analgesic tablet formulated for the Purdue University Student Health Service containing approximately 20 per cent salicylamide and 32 per cent aspirin.

‡ A sodium alginate product.

drugs were suspended in this agent in such a way that the volume of solution administered to the animals remained constant.

Method. Four groups of 12 animals each were selected for the antipyretic studies, and subdivided into groups of four and identified. Twelve animals were used for each test. Of these, four served as non-fevered controls receiving no drug nor fevering agent, four as fevered controls receiving fevering agent only and the remaining four received fevering agent and the specific test dose of a drug four hours after the administration of peptone (fever peak).

The large groups of 12 animals were crossed-over, each group being used every fourth day. The smaller groups were also crossed-over so that each would serve at one time or another as non-fevered controls, fevered controls or receive fevering agent and the drug. This cross-over method permitted all animals to serve as their own controls, thus minimising individual variation, was more exemplary of a population and permitted sufficient rest and recovery from the drug and fevering agent. Fatalities were not observed and all animals appeared to thrive during the duration of the tests.

The fever responses to peptone from a group of eight to 20 rats, at the four hour (peak fever) and seven hour intervals upon three successive tests (two weeks apart) were statistically compared. At both time intervals no significant differences in fevered temperatures for the three successive tests were found (P range = 0·3 to 0·5). The errors and tests for significance were calculated by Students method of "*t*". Hence, neither the intensity nor the duration of the fever induced by peptone injections was significantly altered when the rats were repeatedly used.

In determining plasma-salicylamide levels the method of Keller[12], as modified by Cosmides[13] was used. Animals were again rendered febrile as in the antipyretic studies, drug introduced orally at peak fever, four hours after introduction of peptone, and blood withdrawn by cardiac puncture at specific time intervals after administration of the drug. Heparin 0·1 ml. served as the anticoagulant. Approximately 5 ml. of blood was withdrawn from each etherized animal and the animal subsequently killed.

Results

At completion of the tests the recorded temperatures of fevered and non-fevered controls were averaged. Figure 1 shows the resultant temperature curves. The curves from 4–7 hours in Figure 1 are used as control curves in Figures 2–5.

Figure 2 shows the effect of 100, 200, 250 and 300 mg./kg. of acetylsalicylic acid. The three higher doses exert similar antipyretic effects and reduce peptone-induced fever to approximately the normal level. For this reason, the 200 mg./kg. dose was employed for subsequent comparisons. Salicylamide in doses ranging from 50–300 mg./kg. failed to demonstrate a sustained decrease in temperature. Maximum decrease was observed at approximately 30 minutes after drug administration and rapidly rose thereafter (Fig. 3). These results confirm those reported by

Bavin and colleagues[7]. The 300 mg./kg. dose was observed to maintain a somewhat longer duration of activity.

Fever reductions observed with combinations of salicylamide and aspirin failed to demonstrate a significant difference at peak activity from those seen with aspirin alone (Fig. 2, 4 and Table II). At the end of two hours the animals receiving 200 mg./kg. of aspirin had a significantly lower temperature than those receiving 100 mg./kg. each of aspirin and

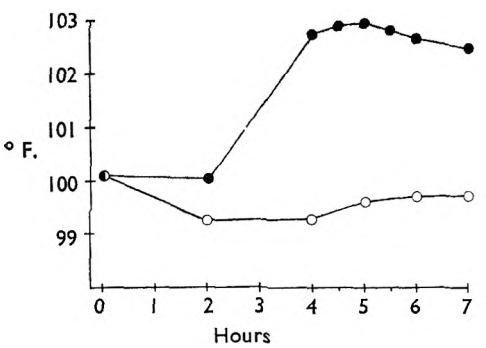


FIG. 1. Comparison of fevered and non-fevered controls.

● Fevered controls.
○ Nonfevered controls.

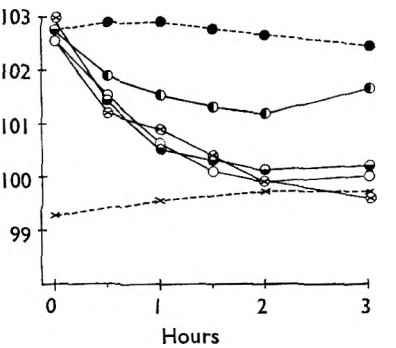


FIG. 2. Effects of acetylsalicylic acid upon oral introduction to peptone pyrexic rats. Fevered controls, ●; nonfevered controls ×; acetylsalicylic acid in mg./kg., ◐, 100; ◒, 200; ○, 250; ⊕, 300.

salicylamide (Table III). Comparison of the effects of the P-tablet with aspirin and salicylamide may be observed in Figure 5. It can be readily seen that the P-tablet has less than half the antipyretic activity of aspirin ($P < 0.01$, see Table II). Aspirin 200 mg./kg. was shown to be a more potent antipyretic than 100 and 200 mg./kg. of salicylamide and 200 mg./kg. of the P-tablet* (Table II). The antipyretic activity of 300 mg./kg. of salicylamide and aspirin-salicylamide, 100 mg./kg. of each,

TABLE I

COMPARISON OF THE PLASMA-SALICYLAMIDE LEVELS IN MG. PER CENT AFTER ORAL ADMINISTRATION OF 200 AND 300 MG./KG. TO PEPTONE PYREXIAL RATS*

		Minutes			
	Normal	5	10	20	30
Mg. per cent after 200 mg./kg.	6·54	16·00	12·54	9·37	9·36
Standard error	±0·71	± 0·24	± 0·89	± 0·55	±1·24
Mg. per cent after 300 mg./kg.	6·54	13·70	14·28	10·80	9·43
Standard error	±0·71	± 1·40	± 2·22	± 0·81	±1·13

* Between 5 and 13 animals used at each time interval.

were not significantly different when compared to aspirin at peak activity (Table II), however, at the end of two hours they both exhibited significantly less effects than aspirin (Table III).

* Equivalent to approximately 39 mg./kg. of salicylamide and 64 mg./kg. of aspirin.

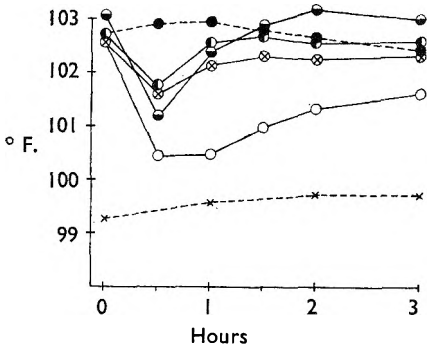


FIG. 3. Effects of salicylamide upon oral introduction to peptone pyrexic rats. Fevered controls, ●; nonfevered controls, ×; salicylamide in mg/kg., ⊕, 50; ◐, 100; ◒, 200; ○, 300.

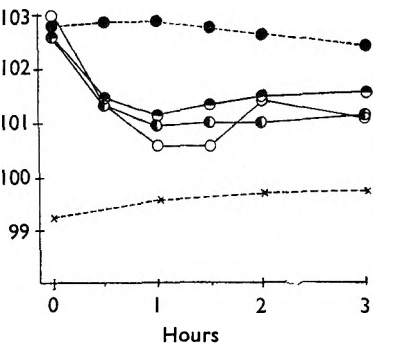


FIG. 4. Comparison of the effects of acetylsalicylic acid-salicylamide combinations upon oral introduction to peptone pyrexic rats. Fevered controls, ●; nonfevered controls, ×; acetylsalicylic acid-salicylamide combinations in mg./kg. of each, ◓, 25; ◐, 50; ○, 100.

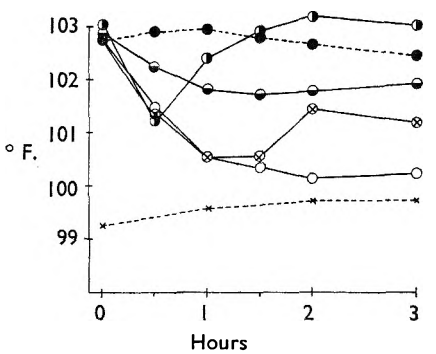


FIG. 5. Comparison of the effects of the P-tablet, acetylsalicylic acid and salicylamide upon oral introduction to peptone pyrexic rats. Nonfevered controls, ×; fevered controls, ●; acetylsalicylic acid 200 mgl/kg., ○; salicylamide 200 mg./kg., ◑; acetylsalicylic acid-salicylamide combination 100 mg./kg. of each, ⊕; P-tablet 200 mg./kg., ◒.

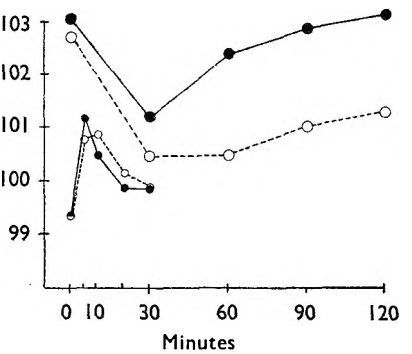


FIG. 6. Comparison of the plasma-salicylamide levels to salicylamide antipyresis after oral administration of 200 and 300 mg./kg. doses to peptone pyrexic rats. Salicylamide in mg./kg., ●, 200; ○, 300. The ° F. scale between 99 and 103° F. also corresponds to plasma levels of 5, 10, 15, 20 and 25 mg. per cent.

Plasma-salicylamide levels were determined with 200 and 300 mg./kg. doses (Table I). The peak concentration was observed five minutes after administration with 200 mg./kg. and at 10 minutes with 300 mg./kg. The differences in concentrations at 20 and 30 minutes are insignificant. Figure 6 shows a comparison between antipyretic values obtained with 200 and 300 mg./kg. doses and the plasma-salicylamide levels obtained with the same doses. The reflection of high plasma-salicylamide levels on pyrexia is not immediately manifested, and its short antipyretic quality appears to be related to the rapid disappearance of the free salicylamide from the blood.

TABLE II

A COMPARISON OF PEAK ANTIPYRETIC ACTIVITY OF ASPIRIN WITH SALICYLAMIDE AND ASPIRIN-SALICYLAMIDE COMBINATIONS

Drug	Peak activity time* hours	No. of animals	Dose mg./kg.	Fever reduction	SE	P
Aspirin	2	8	200	2·6 ±	0·17	—
Salicylamide	0·5	8	100	1·1 ±	0·33	<0·01
Salicylamide	0·5	8	200	1·7 ±	0·36	<0·05
Salicylamide	0·5	8	300	2·4 ±	0·32	>0·5
Aspirin/Amide†	1·5	8	200	2·3 ±	0·29	>0·5
P-Tablet‡	1·5	16	200	1·1 ±	0·17	<0·01

* Calculated from the time of introduction of drug at peak fever (4 hours after peptone injection.)
† 100 mg./kg. of each.
‡ An analgesic tablet formulated for the Purdue University Student Health Service.

TABLE III

A COMPARISON OF THE ANTIPYRETIC ACTIVITY OF ASPIRIN WITH SALICYLAMIDE AND ASPIRIN-SALICYLAMIDE COMBINATION AT 2 HOURS*

Drug	No. of animals	Dose mg./kg.	Fever reduction	SE	P
Aspirin	8	200	2·6 ±	0·17	—
Salicylamide	8	300	1·4 ±	0·41	<0·05
Aspirin/Amide†	8	200	1·2 ±	0·42	<0·02

* Termination of test.
† 100 mg./kg. of each.

DISCUSSION

Acetylsalicylic acid is shown to be superior to aspirin-salicylamide combinations, to salicylamide alone, and to the P-tablet.

The antipyretic effect of salicylamide is in conformity with that reported by Bavin and colleagues[7]. Initial onset is rapid, but duration is very short. Consideration of the blood levels obtained through colorimetric readings supports the belief that the antipyretic activity of this compound is due to the unconjugated form.

Seeberg and associates[14] report salicylate concentrations in the brain to be twice as high with salicylamide as with aspirin when administered orally to rabbits in 500 mg./kg. doses. Peak concentration was demonstrated at one half-hour after dosing. The concentration fell while that of aspirin gradually rose until the curves crossed three hours after introduction of the compounds. They report further that concentrations of salicylamide in other body tissues is equal to that found in the serum 10 minutes after intravenous introduction of 50 mg./kg. in rabbits. Protein-binding was found to occur much more rapidly with aspirin than with salicylamide. They conclude that the low serum concentrations elicited with salicylamide is not due to protein-binding, but to rapid diffusion throughout the body tissues. It appears, then, that antipyresis is dependent on the presence of free salicylamide in the blood.

Combinations of aspirin and salicylamide are shown to be less effective than those of aspirin alone. Therefore there is no apparent potentiation between the compounds. The P-tablet demonstrates an antipyretic curve similar to that seen with aspirin, but to a much lesser degree. This may be due to the presence of caffeine in the formula[11]. Comparisons of the two

active ingredients of the P-tablet indicates that the antipyretic activity is largely due to the presence of aspirin.

Summary

1. Aspirin is shown to be superior to aspirin-salicylamide combinations, salicylamide alone, and to the P-tablet as an antipyretic.

2. The antipyretic effect of salicylamide is rapid in onset, but short in duration.

3. Comparison of plasma-salicylamide levels to antipyresis indicates that the activity of this compound as an antipyretic is probably due to the unconjugated, free amide.

References

1. DeVaney, *The Formulation of an Analgesic Tablet*, Purdue University Library, 1954.
2. Hofmann and Neubauer, *Dtsch. Gesundheitsw.*, 1950, **5**, 776.
3. Holtz and Drebinger, *Schweiz. med. Wschr.*, 1950, **80**, 1175.
4. Euler and Remy, *Med. Klin.*, 1950, **45**, 1178.
5. Lechleitner, *Dtsch. med. Wschr.*, 1951, **76**, 1303.
6. Drebinger, *Die Medizinische*, 1952, **7**, 795.
7. Bavin, Macrae, Seymour and Waterhouse, *J. Pharm. Pharmacol.*, 1952, **4**, 872.
8. Matsumura, *Folia Pharmacol. Japon.*, 1954, **50**, No. **1**, 605.
9. Carlsson and Magnusson, *Acta pharm. tox., Kbh.*, 1955, **11**, 248.
10. Hart, *J. Pharmacol.*, 1947, **89**, 205.
11. Gibson, Miya and Edwards, *J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 605.
12. Keller, *Amer. J. clin. Pathol.*, 1947, **17**, 415.
13. Cosmides, Stemler and Miya, *J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 16.
14. Seeberg, Hansen and Whitney, *J. Pharmacol.*, 1951, **101**, 275.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Adrenaline and Noradrenaline, Fluorescence of, with Ethylenediamine. G. P. Burn and E. O. Field. (*Nature, Lond.*, 1956, **178**, 542.) Weil-Malherbe and Bone have estimated the relative proportions of adrenaline and noradrenaline in mixtures of the two by the different colours of fluorescence given by these substances when coupled with ethylenediamine and extracted into *iso*butanol. This work investigates further the mechanism of the reaction. The reaction proceeds in several steps and the relative rates of formation and decomposition depend on temperature, pH and concentration of the ethylenediamine. Optimum conditions for the formation of the fluorescent material are pH 9·5, ethylenediamine concentration 0·4 per cent; the yield being maximal in 15 minutes. At higher concentrations of ethylenediamine decomposition is rapid. It is possible to separate both the adrenaline and noradrenaline derivatives into two fractions; having different R_F values and fluorescing a different colour. Evidence from the emission spectrum of the noradrenaline derivative shows that the proportions of the two fluorescent products depend on the concentrations of noradrenaline and of ethylenediamine. As therefore there are at least two fluorescent derivatives of adrenaline and noradrenaline the conditions of the experiment must be very carefully controlled in order to obtain reproducible results. M. M.

Hexachlorophene in Liquid Soaps, Determination of. R. F. Childs and L. M. Parks. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 313.) In alkaline solution, hexachlorophene exists mainly in an ionic form, which has a greater ultra-violet absorption than the unionised form, with shift of the absorption peaks to longer wavelengths. The authors describe a method of assay which depends on measurements of the difference in absorption at 312 mμ between acid and alkaline solutions. A 5 g. sample of hexachlorophene soap is dissolved in ethanol to produce 100 ml. 25 ml. of this solution is diluted to 100 ml. with methanol (90 per cent) and 15 ml. is placed in each of two flasks. To one flask is added 0·3M acetic acid to 50 ml., and to the other, methanol (90 per cent) to 50 ml. The difference between the ultra-violet absorption of these solutions is determined at 312 mμ, and the content of hexachlorophene is calculated from a standard curve showing the difference in absorption of solutions of pure hexachlorophene at pH 8 and pH 3. The absorption due to the presence of soap is the same in both solutions and so does not affect the result. G. B.

Ipecacuanha, Assay of. E. Brochmann-Hanssen. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 344.) Ipecacuanha may be extracted by means of ion exchange resins, using the technique previously described (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 74). 0·2 g. of ipecacuanha is placed in an extraction tube with 1 g. of activated cation exchange resin (Dowex 50-X_2) and 20 ml. of water. After shaking for 15 minutes, the mixture is transferred to a suitable ion exchange tube and the exhausted drug removed by a backwash of distilled water. The alkaloids are eluted with 4N ammonium hydroxide in methanol (70 per cent) and the extract purified by passing it through a column of anion exchange resin

(Dowex 1-X_1) previously activated with N sodium hydroxide. The quantity of non-phenolic alkaloids in solution is determined by measuring the ultra-violet absorption at 285 mμ against a blank solution prepared by passing methanolic ammonia through the same column of Dowex 1-X_1. The result is calculated as emetine. For the determination of phenolic alkaloids the anion exchange resin is washed with water to remove ammonia and the alkaloids eluted with 0·1N acetic acid. After the addition of sodium hydroxide-sodium bicarbonate solution and a coupling agent, the colour intensity is determined at 495 mμ against a blank prepared similarly but without the coupling agent. The result is calculated in terms of cephaeline. Using this method, 15 minutes at room temperature is sufficient for complete extraction of the alkaloids from the drug. Results are appreciably higher than those obtained by the U.S. Pharmacopeia method, in which inefficient extraction appears to be responsible for the low results. The proposed method is simple, rapid and capable of high precision.

G. B.

Local Anaesthetics, Determination of, in Non-aqueous Media. B. Salvesen, S. Kristoffersen and A. Aasbø. (*Medd. Norsk. Farm. Sels.*, 1956, **5**, 88.) Cocaine, amylocaine, pseudococaine, phenacaine and cinchocaine hydrochlorides were titrated in a mixture of equal volumes of glacial acetic acid and dioxan, in the presence of mercuric acetate. Perchloric acid in acetic acid was used as the titrating agent, and a sharp end point was observed when crystal violet was used as indicator. Results close to the theoretical figure were obtained. Procaine, larocaine and amethocaine hydrochlorides gave two inflections of the potential curve, both of which could be used as end points of the potentiometric titration, but the indicator change, corresponding to the second potentiometric end point, was not sharp. The compounds were acetylated by heating to boiling a solution of the local anaesthetic agent in a mixture of 5 volumes of acetic acid with 1 of acetic anhydride, after which the solutions could be titrated as above, and a sharp colour change (violet to blue) obtained with crystal violet as indicator. G. B.

Thiamine and Riboflavine in Mixtures, Fluorimetric Determination of. W. E. Ohnesorge an L. B. Rogers. (*Analyt. Chem.*, 1956, **28**, 1017.) This method was designed to eliminate the necessity for chromatographic separation of the vitamins before analysis. The thiamine (aneurine) is converted to thiochrome by alkaline ferricyanide, the pH is adjusted to such a value that both the riboflavine and thiochrome fluoresce. The exciting radiation consists of a monochromatic source at 365 mμ, and the fluorescence at 450 mμ (chiefly due to the thiochrome) and at 530 mμ (chiefly due to the riboflavine) is measured. The fluorescent spectra overlap but calibration charts are prepared using known mixtures of the vitamins from 0·5 to 4·0 p.p.m. of each and a quinine standard for arbitary comparison. The standard deviations are of the order of $\pm$ 5 per cent for each vitamin. The effect of a number of variables such as pH, temperature, time of irradiation and the effect of alkaline ferricyanide and light on riboflavine fluorescence are considered. D. B. C.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

Digitalis Leaves, Biological Standardisation of. K. B. Jensen. (*Acta pharm. tox., Kbh.*, 1956, **12**, 136.) An investigation has been made of the efficiency of the most commonly employed glycoside extraction procedures used in the standardisation of digitalis, namely maceration with 70 per cent ethanol, soxhlet extraction with absolute ethanol for six hours and maceration with

absolute ethanol for 24 hours followed by boiling on a water bath for 30 minutes. Paper chromatographic and fluorimetric determinations were made of the individual cardio-active glycosides and aglycones, and the results were compared with a percolation procedure using 70 per cent ethanol previously shown to give total extraction. It was found that maceration with 70 per cent ethanol gave total extraction of the cardio-active substances, but the two methods with absolute ethanol gave very incomplete extraction. G. F. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Adrenaline and Noradrenaline Concentrations in Rat Tissues, Seasonal Variations of. K. A. Montagu. (*Nature, Lond.*, 1956, **178**, 417.) Variations of catechol amine concentration in tissues from male rats were studied over a period of 12 months. Adrenaline and noradrenaline were estimated fluorimetrically in the heart, kidneys, liver, diaphragm, leg muscles, brain and spleen, using a modification of the method of Weil-Malherbe and Bone. The adrenaline and noradrenaline concentrations and the percentage of adrenaline in the mixture usually showed two maxima and two minima in the year, often of very unequal size. Both the amines showed a 2–5 fold variation in the different months, the maximum concentrations being in the summer and winter and the minimum in spring and late summer. The percentage of adrenaline showed 1·5–3 fold variations, being maximum in March and July. These fluctuations were true of all the tissues except brain. The variations in the heart, liver, leg muscles and spleen were statistically significant. Experiments with bilaterally demedullated rats indicated that the variations are not dependent on the adrenal medulla. They cannot be due to seasonal differences of environmental temperature or diet, nor solely to differences of light nor to interconversion of the two amines. They might have been caused in part by differences in the weight of tissues expressed as percentages of body weight; seasonal metabolic changes might account for changes of weight and of the adrenaline and noradrenaline contents of tissues. M. M.

Amine Contents of the Two Adrenal Glands of the Cat, Relation between. K. R. Butterworth and M. Mann. (*Nature, Lond.*, 1956, **178**, 363.) Since frequently there is a difference in the weight of the left and right adrenal glands of the cat a statistical investigation is carried out into the relation between the two glands of each animal. The following facts were investigated: (1) the relative percentages of noradrenaline in the two glands of each animal and (2) whether the total amine content is the same for both glands or whether the heavier gland contains more amine. The glands from each of 36 adult cats were dissected out and 50 mg./ml. extracts prepared in 0·1 N hydrochloric acid. The adrenaline and noradrenaline content of each extract was determined biologically, using the cat blood pressure and nictitating membrane as separate test preparations and calculating the results by the formula of Bülbring. It was found that the percentages of noradrenaline in the left and right glands of each animal were the same. The mean percentage of noradrenaline in the left gland was 44·5 per cent and in the right gland 44·0 per cent. This correlation between the pairs of glands was independent of the degree of methylation and of any difference in the weight of the two glands. There was found to be a much closer agreement between the amount of amine in the two glands when the results were expressed as per gland than when expressed as per unit weight

of gland. This was true whether the difference in the weight of the two glands was small or large. Differences in the weight of the two glands were as large as 36·4 per cent. These results are of value in experiments where one gland of the animal is used as a control and the effects of drugs on the other are studied.

M. M.

BIOCHEMICAL ANALYSIS

Barbitone in Blood, Quantitative Determination of. R. Askevold and F. Løken. (*Scand. J. clin. lab. Invest.*, 1956, **8**, 1.) A simple extraction method for the determination of barbitone in blood serum is described. It is based on the extraction of the barbiturate from the serum into chloroform through simultaneous removal of water and proteins by addition of sodium sulphate. Pipette 15 ml. of chloroform into a test-tube, add 2 g. of sodium sulphate, 0·1 ml. of concentrated hydrochloric acid and 3 ml. of serum. Shake, add another 2 g. of sodium sulphate and shake again. The chloroform layer should now be free from water. Prepare also a blank in the same way omitting the serum. Filter both sample and blank, pipette 5 ml. of the filtrate into glass stoppered test-tubes and add 5 ml. of borate buffer (pH 10). Shake for 3 minutes, centrifuge and transfer about 3 ml. of the aqueous phase of the sample and the blank into quartz cells. Read in a spectrophotometer at 239 mμ. Add 0·05 ml. concentrated hydrochloric acid to the two, mix and read again.

If spectrophotometer reading at 239 mμ alkaline = D_1
and at 239 mμ acid = D_2

$(D_1 - D_2) \times 10{\cdot}6$ = mg. barbitone sodium/100 ml.

The recovery of barbitone sodium averaged 96 per cent. G. F. S.

Magnesium in Serum, Estimation of. D. W. Neill and R. A. Neely. (*J. clin. Path.*, 1956, **9**, 162.) The titan yellow method for estimating serum magnesium has been modified by the addition of calcium to the standard solution to eliminate any calcium effect, and by the use of gum ghatti in place of hydroxylamine as a colour stabilizer. For the assay, 1 ml. of serum is diluted with 5 ml. of water and proteins are precipitated by the addition of 2 ml. of 10 per cent sodium tungstate and 2 ml. of 0·67N sulphuric acid. After centrifuging take 5 ml. of the supernatant and add 1 ml. of water, 1 ml. of 0·1 per cent gum ghatti solution, 1 ml. of 0·05 per cent titan yellow and 2 ml. of 4N sodium hydroxide solution. Read the optical density in a colorimeter with a 624 filter and determine the magnesium concentration by reference to a standard curve. A blank is run alongside, using 1 ml. of calcium chloride solution (equivalent to 0·05 mg. $CaCl_2$). The normal adult magnesium level in serum by this technique was 2·3 mg. per 100 ml. (range 1·9 to 2·7). G. F. S.

CHEMOTHERAPY

Antibacterial Substances in Seeds. L. Ferenczy. (*Nature, Lond.*, 1956, **178**, 639.) During investigations of the physiology of germination, it was observed that micro-organisms occurring on some species of seeds proliferated during germination whilst seeds of other species remained sterile. It was inferred that antibacterial substances must be released from the seeds during germination. Further investigations were made by the agar diffusion method using the following test organisms: *B. mycoides*, *B. megaterium*, *B. subtilis*, *Staph. aureus*, *A. aerogenes*, *Shig. flexneri*, *Erwinia carotovora* and *Xanthomonas malvacearum*. All were rapidly growing strains, so that sterilisation of the seeds was unnecessary.

Bouillon agar (pH 7·0) plates of 5 mm. thickness were seeded with freshly prepared suspensions, the surfaces of the plates dried and the seeds sunk into the medium. Zones of inhibition were measured after incubation for 20 hours at 30°. Entire seeds were tested with the exception of seeds of species of *Fraxinus*, which were cut transversely in order to ascertain whether germination inhibiting compounds known to be released under these conditions possessed antibacterial properties. Of 400 species examined, the seeds of 36 plant species gave positive results. In most cases the effect was selective, only the Gram-positive bacteria being inhibited. Substances released from *Fraxinus* species were effective against all the test organisms. Seeds of several species of *Kniphofia* were highly active against Gram-positive organisms and the authors report that the active substance from this genus has been isolated. Isolation of antibacterial substances from other seeds has been commenced. B. A. W.

PHARMACY

Cyanocobalamin in the Presence of Aneurine and Nicotinamide, The Stability of. J. Dony and J. Conter (*J. Pharm. Belg.*, 1956, **11**, 186). Solutions containing cyanocobalamin (5 μg./ml.) alone and with various quantities of nicotinamide, aneurine, and both nicotinamide and aneurine together, were prepared and adjusted to pH 5. Samples were kept in sealed ampoules and stoppered bottles, protected from light, while others were subjected to heat treatment. The cyanocobalamin content was then determined by microbiological assay. At ordinary temperatures, this vitamin was stable for up to 2 months, alone or in the presence of aneurine, nicotinamide or both. At 37° it was stable in the presence of either aneurine or nicotinamide, but when both were present together, the stability decreased as the proportion of aneurine was increased. Cyanocobalamin was stable to heat at 100° for 4 hours in the presence of nicotinamide or of up to 10 mg./ml. of aneurine. On autoclaving solutions of cyanocobalamin alone or with nicotinamide at 120° for 20 minutes, a very slight decomposition occurred. Solutions containing cyanocobalamin, nicotinamide and aneurine could not be autoclaved without considerable loss of vitamin B_{12}. G. B.

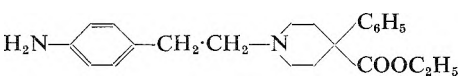
PHARMACOLOGY AND THERAPEUTICS

Acenocoumarin as an Anticoagulant. F. J. Schilling and O. R. Kruesi. (*Amer. J. med. Sci.*, 1956, **231**, 558.) Acenocoumarin (Sintrom) is nitro-phenyl-acetyl-ethyl-4-hydroxycoumarin. It is a white, crystalline, tasteless powder which is administered by mouth as tablets or capsules. This report is based on observations made on 65 patients with thromboembolic conditions who were treated with acenocoumarin for an average period of 27 days. The effective doses were found to be 20 mg. the first day, 8 to 16 mg. the second day, and an average of 4 to 6 mg. daily for maintenance therapy. All the patients developed adequate hypoprothrombinaemia as a result of this therapy. The administration of a single daily oral dose produced an adequate hypoprothrombinaemia which appeared to be well sustained according to daily prothrombin values. On single daily doses of acenocoumarin the 65 patients were within the therapeutic range of hypoprothrombinaemia for about 75 per cent of the time. Excessive hypoprothrombinaemia developed in 3 patients during maintenance therapy, but was successfully treated with vitamin K_1. After cessation of acenocoumarin administration the dilute prothrombin time rapidly

approaches normal within 30 hours. There was no evidence in the series that the drug caused gastrointestinal irritation, or hepatic, renal or haemopoietic toxicity. S. L. W.

Adrenaline and Noradrenaline in Adrenal Autografts. O. Eränkö. (*Nature, Lond.*, 1956, **178**, 603.) Both cortical and medullary cells survive in adrenal grafts after transplantation into the anterior chamber of the eye. An investigation is carried out into whether adrenaline and noradrenaline are present in separate cells in these transplants as is so in the adrenal medulla. Using adult rats, a piece of adrenal medulla is inserted into the anterior chamber of the eye. 5 months later the animals are killed and the grafts removed. The adrenaline and noradrenaline contents of these grafts are estimated chemically after separation of the two amines by paper chromatography. Both amines were detected in the grafts, the adrenaline content being 5–10 times the noradrenaline content. Histological examination of the grafts showed chromaffin cells attached to both the cornea and iris. The cells were closely packed but regular cell acini, such as is present in the adrenal medulla, were replaced by irregular groups of cells. The iodate reaction, which stains the noradrenaline-containing cells brown, was positive in some graft cells but the majority, although showing the chromaffin reaction, remained colourless after iodate treatment, thus indicating the presence of adrenaline-containing cells. This is in good agreement with the chemical observations made and suggests that the grafted adrenomedullary cells have retained their ability to make and secrete the same catechol amine which they were making and secreting in the adrenal before grafting. M. M.

Analgesic, A New Synthetic. J. Weijlard, P. D. Orahovats, A. P. Sullivan, G. Purdue, F. K. Heath and K. Pfister, 3rd. (*J. Amer. chem., Soc.*, 1956, **78**, 2342.) Ethyl 1-(4-aminophenylethyl)-4-phenylpiperidine-4-carboxylate (I) was prepared by condensing *p*-aminophenylethyl chloride hydrochloride with ethyl 4-phenylpiperidine-4-carboxylate "carbonate" in



(I)

ethanol in the presence of sodium bicarbonate. It is a potent analgesic with high oral activity and relatively mild side reactions. Mild anti-acetylcholine and antihistaminic activity has been observed both with isolated organs and in intact animals. In animals the compound approaches morphine in analgesic potency, and does not produce nausea, vomiting or constipation. The acute oral and subcutaneous toxicity in mice is simillar to that of pethidine. The synthetic *N*-acetyl derivative has analgesic activity in rats of the same order as I. Preliminary results on man by oral and parenteral administration indicate an analgesic potency for I at least twice that of pethidine. A. H. B.

Atropine-like Compounds, Relation between the Structure and Action of. M. Ya. Mikhel'son, A. S. Artem'ev, I. V. Dardymov, É. V. Zeĭmal, F. V. Pevzner, E. K. Rozhkova, R. S. Rybolovlev, N. V. Savateev, Ya. R. Savinskiĭ, E. P. Uspenskaya, N. V. Khromov-Borisov, K. G. Tsirk and A. M. Yanovitskaya. (*VIII Vsesoyuznyi s'ezd fiziologov, Biokhimikov, farmakologov*, 1955, 424–426; *Sovetskoe Med. Referat. Obozrenie*, 1956, No. 26,

131–132.) Results of studies on the hydrochlorides, methiodides and ethiodides of diethylaminoethyl phenyl*cyclo*pentancarboxylate ("Pentafen") and diethylaminoacetylphenothiazine phenyl*cyclo*pentancarboxylate ("Difazin") are reported. All exhibited a marked nicotine and a weak muscarine antagonism. The intravenous doses needed to reduce the depressor effect due to excitation of the vagus nerve were one-fifth to one-twentieth of the doses that reduce the depressor effect following intravenous administration of acetylcholine. When the nitrogen of Pentafen or Difazin is converted to the quaternary form, the blocking action on the peripheral acetylcholine receptors is increased ten-fold. Difazin and Pentafen methiodides reduce the depressor action due to stimulation of the vagus nerve in doses of 0·05 mg./kg.; the corresponding dose of the hydrochlorides is 1·5 to 2 mg./kg. In addition, the alkyl iodide compounds show an enhanced capacity for blocking conduction at sympathetic ganglia; they also block the acetylcholine receptors of the suprarenal and carotid synapses; and they reduce the effect of acetylcholine on the blood pressure of the cat, on the isolated frog heart and on the isolated cat gut. The capacity for reversing neostigmine bronchospasm in cats is increased 20 to 50 times by quaternisation.

E. H.

Azacyclonol (Frenquel) in Chronic Schizophrenia. J. T. Ferguson. (*Antibiotic Med.*, 1956, **3**, 146.) The effects of administration of azacyclonol were observed on 264 chronic, hospitalised women mental patients, predominantly schizophrenics. Beneficial reports recorded by other investigators were confirmed. In those schizophrenic patients helped by the drug the primary effect appeared to be on the delusional system, and increases or decreases in psychomotor activity appeared to be secondary to the changes in delusional activity. Quiet, withdrawn patients became more active, while overactive patients became less active. Early in the course of treatment there may be cyclic changes in behaviour, but with continued treatment a return to the old behaviour pattern becomes less frequent. The clinical course was frequently found to be improved when reserpine or methyl phenidate (Ritalin) was administered concurrently with azacyclonol. The optimal oral dose of azacyclonol varies with different patients, but is usually about 100 mg. daily; the intravenous dose is 100 mg. three times daily. The most rapid response is obtained by giving azacyclonol intravenously for 1 to 3 days followed by oral therapy. Encouraging preliminary results were obtained with the drug in the treatment of psoriasis and arthritis occurring in psychotic patients. In this series of 264 patients no gross change in pulse, respiration, temperature or blood pressure was observed with oral doses up to 360 mg./day and intravenous doses up to 300 mg./day. No significant changes were found in non-protein nitrogen values, cephalin-flocculation tests, blood-sugar levels and urinalyses; no abnormalities of the total blood picture were reported.

S. L. W.

Carbutamide (BZ55) – Experimental and Clinical Studies. (*Canada med. Ass. J.*, 1956, **74**, 957–998.) This is a series of preliminary reports on work carried out on this oral antidiabetic in the laboratories and clinics of Toronto and presented during recent months at meetings of the Toronto Diabetes Association. The symposium, which consists of 7 experimental and 10 clinical reports, is prefaced by a short article by C. H. Best on insulin adjuvants or substitutes in which he concludes that while carbutamide would appear to be therapeutically effective in selected cases its widespread clinical use should only be considered after prolonged experimental and clinical research. In the experimental

reports, evidence is presented to show that carbutamide stimulates the islets of Langerhans, reduces the rate of absorption of glucose from the gut, and reduces the hexose-6-phosphatase activity of the liver, influencing glucose liberation from that organ. Each of these three changes would tend to reduce the blood-sugar level. Carbutamide is shown to be effective in lowering the blood-sugar level in depancreatised and in Houssay dogs, maintained with exogenous insulin, thus indicating that neither the pancreas nor the pituitary gland is essential for the action of the drug; carbutamide appeared to potentiate the action of insulin. On the other hand, on withdrawal of insulin, carbutamide did not prevent glycosuria in the pituitary-diabetic dog, nor did it prevent glycosuria, polyuria, ketonuria, and the rapid onset of severe upset in the depancreatised dog. No large-scale series of trials are included in the clinical reports, a total of only 41 cases being dealt with in the 10 papers. These preliminary trials seem to indicate that carbutamide has a limited value in controlling mild cases of diabetes, that is, in those cases which may be presumed to have reasonable amounts of endogenous insulin, but that it is unlikely to be of value in severe cases. It was of no value in lowering the blood sugar level in a patient with pancreatic diabetes following pancreatectomy, who had been successfully controlled for 5 years by insulin injections. Neither was it found of value in any one of 5 children whose diabetes was successfully controlled by insulin injections. Three of the children treated with carbutamide developed a blotchy, erythematous rash with pruritus, and one developed a mild febrile reaction as well. One other case in this series of trials developed an erythematous, pruritic rash, but no other toxic effects are reported. S. L. W.

Carbutamide (BZ55) in Diabetes, Clinical Trial of. J. A. Hunt, W. Oakley and R. D. Lawrence. (*Brit. med. J.*, 1956, **2**, 445.) Carbutamide was tried in a representative group of 21 patients selected to cover a wide range of age, weight, severity and duration of diabetes; on the basis of the result a group of 17 were selected for treatment as out-patients. Diet control consisted of measured carbohydrate with free protein and fat. The patients were first studied for a control period of two weeks during which they were given dummy tablets. On the first and second days of the trial they were given 2·5 g. and 1·5 g. of the drug; subsequently the dose was 1 g. daily. If a satisfactory response was not obtained in 7 days, the maintenance dose was increased. In the representative group, glycosuria disappeared completely in 9, decreased in 6 and was unaffected in 6. Of the 15 who showed a response, the age at onset of diabetes varied from 45 to 75; only 4 of them had required insulin. Patients with heavy ketonuria did not respond to the drug. In the selected group the diabetes developed after the age of 40; none showed significant ketonuria without insulin. Glycosuria was not controlled by diet alone but in all cases it was reduced or eliminated when carbutamide was given. In the 8 patients who had previously been receiving insulin, the dosage varying from 12 to 88 units, the response was good in 4 and moderate in 4. From the results in the representative group it was concluded that young diabetics and those requiring more than small amounts of insulin show little or no response and there was no reliable evidence that carbutamide reduced the insulin requirement; in two young diabetics the glycosuria increased when the carbutamide was given in addition to their normal dose of insulin. Toxic reactions included giddiness in 8 patients, in 5 of whom it occurred only after the first dose of the drug. In 2 cases there was an irritant erythematous rash which disappeared on stopping treatment. One patient had a sulphonamide rash and one had a sulphonamide drug fever. H. T. B.

Hyperfibrinogenaemic Action of (+)- and (−)-Adrenaline. F. Mandelbaum, O. B. Henriques and S. B. Henriques. (*Nature, Lond.*, 1956, **178**, 363.) Studying the hyperfibrinogenaemic effect of several sympathomimetic amines, it was found that adrenaline, when given subcutaneously to rats, had the highest activity in increasing the blood plasma fibrinogen. Comparing the activity of (+)- and (−)-adrenaline, 40 rats were randomly distributed into 4 groups and given suitable doses of either isomer. 24 hours later the animals were bled under suitable conditions and the fibrinogen content determined by the standard technique of Cullen and Van Slyke. The relative potency of the two isomers was calculated by standard statistical methods. The results indicate that the *dextro* isomer has 2·8 per cent of the activity of the *laevo* isomer. This ratio is comparable to the ratio found for the pressor response in dogs. M. M.

Isopromedol, Pharmacological Properties of. M. D. Mashkovskii and P. N. Abramova. (*Farmakologiya i Toksikologiya*, 1956, **19**, No. 3, 26–32.) A pharmacological study of isopromedol, a stereoisomer of promedol (1 : 2 : 5-trimethyl-4-phenyl-4-piperidyl propionate) is reported. Isopromedol has m.p. 181° to 182·5° and the m.p. of its piperidol is 102° to 103°; the m.p. of the piperidol corresponding to promedol is 107° to 108°. Using white rats, rabbits and dogs, isopromedol was shown to be 2 to 3 times as analgesic as promedol, with similar properties; its effect is more prolonged. It is more powerful than promedol in relaxing smooth muscle. The toxicity of the two isomers is about the same. Isopromedol has been tried in 600 various gynaecological-obstetric patients; it is given subcutaneously in 1 to 2 per cent solution in 1 ml. doses or, less frequently, in oral doses of 0·025 to 0·05 g. The preparation has been approved for clinical use by the Scientific Council of the USSR Ministry of Health. E. H.

Lysergic Acid Diethylamide, Some Serotonin-like Activities of. E. Shaw and D. W. Woolley. (*Science*, 1956, **124**, 121.) Lysergic acid diethylamide (LSD) has been shown on the isolated heart of the clam (*Venus mercenaria*), and on the blood pressure of the dog, to have a serotonin-like action preceding an antagonism. In the anaesthetised dog, LSD showed, like serotonin, both pressor and depressor effects. The pressor effect was 1 to 3 times as powerful as serotonin and could be prevented by the new powerful antagonist of serotonin, 1-benzyl-2:5-dimethylserotonin. It is suggested that LSD, because of its structural resemblance to serotonin, combines with the receptors causing an initial stimulation prior to an antagonistic block. G. F. S.

Novobiocin in Pneumonia. B. M. Limson and M. J. Romansky. (*Antibiotic Med.*, 1956, **2**, 277.) Novobiocin was effective in the treatment of 30 patients with bacterial pneumonia, the results appearing comparable to those with other antibiotics. The fever subsided within 24 to 48 hours in 16 patients, 72 to 96 hours in 10, 5 days in 2, and 7 days in 2. Resolution of the pneumonia was noted within 3 to 4 days in 7 patients, 5 to 7 days in 13, 8 to 11 days in 9, and 14 days in 1. The total amount of antibiotic administered ranged from 6 to 24 g., the majority of patients receiving 12 to 16 g. High serum concentrations were obtained following cumulative oral administration of 500 mg. of novobiocin every 6 hours. Side reactions were limited to 2 cases of mild urticaria which cleared up within 48 hours after stopping the antibiotic. *In vitro* sensitivity tests of 18 strains of pneumococci isolated from patients showed all to be sensitive to less than 0·4 μg./ml. of novobiocin. S. L. W.

(ABSTRACTS *continued on p.* 144.)

BOOK REVIEW

A TEXTBOOK OF PHARMACOGNOSY, by N. M. Ferguson. Pp. vii + 374 (including Index). The Macmillan Company, New York and London. 1956. 42s.

The list of subjects dealt with in this book illustrates the change in emphasis which Pharmacognosy is undergoing. Most of the research effort of previous generations of pharmacognosists was directed to describing the structural features of the rather large number of vegetable and animal drugs being used as such, in order that correct identification and freedom from adulteration could be established. This research work has resulted in a large and useful body of information, which, on the whole, is of the sort that will not need much further modification. Of recent years, however, pharmacognosists have taken an increasing interest in the constituents of crude drugs; this accords with Flückiger's definition of the scope of Pharmacognosy (quoted by Ferguson in his Introduction) as "the simultaneous application of various scientific disciplines with the object of acquiring the knowledge of drugs from every point of view". The present shift in emphasis or point of view is from the study of the *container* —the crude drug—to the study of the *contents*—the constituents. This includes the study of their location, biosynthesis and role in the living plant, and their properties with a view to more precise methods of evaluating and separating the pharmacologically useful constituents. In accordance with this trend, the author starts with a general chapter on the biosynthesis of plant constituents and follows this with chapters on various groups of constituents, such as carbohydrates, glycosides, alkaloids and includes such natural products as antibiotics, blood products, allergens, vitamins and hormones. These chapters aim at giving a knowledge of the chemical and physical properties of the constituents, especially in relation to prescription compounding, methods of extraction and the proprietaries containing them. There is practically no information on the macroscopical and microscopical characters of crude drugs. While, as indicated above, research in this branch of Pharmacognosy is not so active as formerly, it is still an important part of the subject, as an essential prerequisite of the study of crude drugs is correct identification. A knowledge of the techniques used in this process should be an important part of the teaching programme. However, the author may have felt that this aspect is adequately covered in other textbooks of Pharmacognosy, though the student is not given any general reference to them.

Unfortunately this very understandable ambition to present the modern aspects of Pharmacognosy has not been fulfilled. The treatment of the subjects throughout the book is very superficial, especially when certain recent developments are dealt with. The important techniques classified under the heading chromatography are dealt with in two pages; the information is sketchy and no illustration of its application in plant analysis is given. An enormous amount of work has been done in the last two decades on alkaloidal biosynthesis, yet this subject is scarcely mentioned. Similarly, there is a very inadequate treatment of recent work on such drugs as rauwolfia, veratrum, curare, *Ammi* species, rutin, alginates, the production of cortisone from plant steroids, etc. Colchicine is mentioned, but no reference is made to its use in genetics and the application of such use in Pharmacognosy. There are a number of errors in the text, a few of which should be mentioned. Ipecacuanha is said to contain not less than 12 per cent of ether soluble alkaloids (p. 14); the main interest in curare is said to be as a possible

muscle relaxant in the treatment of poliomyelitis (p. 227). The delightful story of the Countess of Chinchon's use of cinchona is given, but the author does not point out that recent historical research has shown the story to be false. Similarly, Dioscorides wrote about 100 A.D., not 77 B.C., and his work does not describe several thousand drugs. Perhaps rather naturally, I looked up the information on anthraquinone drugs and, though the author has published research work on this group, there are several serious errors. For instance, senna leaf (p. 97) is said to contain frangula emodin and chrysophanic acid; while it would be difficult to state categorically that this is untrue, so far no research work has proved their presence. Further, the author states that glycosides are only "believed to be present", sennosides A and B being merely "a glycosidal extract". Stoll and others isolated these substances as far back as 1942 and have since shown that they are pure chemicals whose structure has been completely elucidated. There is practically no information given on any recent work on the anthraquinone drugs.

While the author is to be congratulated on a modern approach to the subject, it is to be hoped that a further edition will correct the numerous errors, and give a more thorough treatment of important developments; in such circumstances the book will serve as a valuable guide to the study of one aspect of Pharmacognosy.

J. W. FAIRBAIRN.

(ABSTRACTS *continued from p.* 142.)

APPLIED BACTERIOLOGY

Bile Salts in Culture Media, a Substitute for. J. E. Jameson and N. W. Emberley. (*J. gen. Microbiol.*, 1956, **15**, 198.) The authors report on the use of the anionic detergent Teepol as a substitute for bile salts in culture media for organisms of the coli-typhoid group. It was found that Teepol was more selective against Gram-positive organisms than bile salts and it suppressed swarming of *Proteus* at lower concentrations. Investigation of the composition of Teepol from batch to batch revealed that variations in the relative amounts of alkyl sulphates of differing chain length were small. Teepol contains small residues of unsulphated organic matter, but saturated solutions of this residue in nutrient broth freely supported growth of *E. coli* and *Staph. aureus*. Media (liquid and solidified with agar) containing 0·1 per cent Teepol were compared with MacConkey medium. The Teepol medium tended to precipitate neutral red, and the best results were obtained when bromocresol purple was used as indicator. Plate counts by the Miles and Misra technique of cultures of 12 organisms of the coli-typhoid group were not significantly different when either Lemco, MacConkey or Teepol media were used. Mean colony diameters of 6 organisms were definitely larger when grown on Teepol medium. One or more strains of 12 serotypes each of *Shigella* and *Salmonella* grew freely on Teepol agar, whereas enterococci, micrococci and staphylococci did not grow. Teepol medium was shown to be as effective as MacConkey medium in the isolation of strains of *E. coli* from rectal swabs taken from babies with gastroenteritis and at least as effective for presumptive coli counts taken on 166 drinking waters and 162 sea waters. Colonies on Teepol agar did not readily go rough as on some batches of MacConkey agar and were at least equally suitable for slide-agglutination tests. The authors consider that Teepol is probably of far more constant composition than either sodium taurocholate or tauro-glycocholate and has the added advantage of being much cheaper. B. A. W.