# REVIEW ARTICLE

## THE CHEMOTHERAPY OF MALARIA*

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Man is concerned with only four well-authenticated species, *Plasmodium vivax*, *P.falciparum*, *P.malariae* and *P.ovale*, of some 140 known species, of the protozoon *Plasmodium* which, transmitted by the female anopheline mosquito, are the cause of malaria. There is a high degree of specificity in host-parasite relationships for, with the exception of *Plasmodium vivax* which can produce a mild parasitaemia in chimpanzees[1], none of the human parasites has yet been transmitted to other animals.

### Life-cycle of the malaria parasite

The life-cycle of the malaria parasite is complex and in order to make clear later the roles played by the various antimalarial drugs a brief outline is now given.

With the bite of the infected mosquito, the definitive host, sporozoites from the salivary glands of the insect are injected into the blood stream of the man, the intermediate host, (see Fig. 1). The sporozoites disappear from the peripheral blood within about 30 minutes and schizonts of a pre-erythrocytic stage may subsequently be detected in the parenchymal cells of the liver[2,3]. In the case of *P. cynomolgi*, trophozoites have been detected only one day after intravenous injection of sporozoites into monkeys[4]. The nuclei of the schizonts divide and 8 to 12 days after the mosquito bite the liver cell is ruptured and a large number of merozoites are released, some of which penetrate fresh liver cells and the cycle in the liver is thereby continued. Others invade red blood cells. The first stage—trophozoite—in the blood eventually becomes a schizont which undergoes cell division giving rise to merozoites. These are released when the red blood cell disintegrates and invade further red blood cells. At this point the temperature of the host rises and a bout of fever ensues. The period of time from the initial invasion of the erythrocytes to the release of merozoites is characteristic of the species of parasite. Thus, *P.malariae* which causes quartan malaria, has a blood cycle lasting 3 days, whilst *P.vivax* and *P.falciparum* have a 2-day repeat pattern causing benign and malignant tertian malaria respectively.

During the asexual cycle in the blood, sexual forms appear—the female macrogametocyte and the male microgametocyte. When a mosquito bites at this stage the gametocytes are taken up with the blood meal and within the insect sexual reproduction takes place. A further complicated sequence of events results finally in sporozoites finding their way into the salivary glands and there they await an opportunity to infect another human subject.

* Based on one of a series of lectures on "Chemotherapy" given at The Royal Technical College, Salford, Lancashire, during October and November, 1957.

The exo-erythrocytic cycle in the human liver continues apparently independently of the cycle in the blood and relapses in vivax malaria must be attributed thereto[5]. In the case of *P.falciparum* there appears to be no continuing phase in the liver for relapses of malignant tertian malaria are not known[6].

## EVALUATION OF ANTIMALARIALS

Because of species specificity it is not possible to use the parasites of man in the laboratory for the screening and initial evaluation of potential antimalarial drugs. Avian parasites have been widely employed in this respect and the use of *P.relictum* infections in canaries led to the discovery

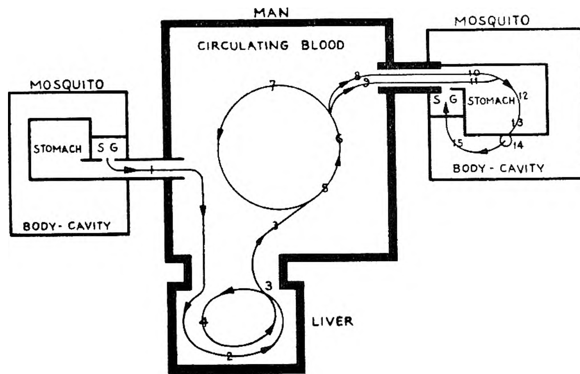



FIG. 1. Life cycle of malaria parasite:

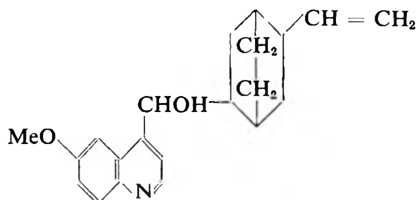
1. Sporozoites
2. Pre-erythrocytic schizonts (cryptozoites)
3. Merozoites
4. Exoerythrocytic schizonts (phanerozoite, late exo-erythrocytic forms)
5. Erythrocytic trophozoites
6. Erythrocytic schizonts
7. Erythrocytic merozoites
8. Macrogametocytes
9. Microgametocytes
10. Macrogametes
11. Microgametes
12. Zygotes
13. Ookinetes
14. Oocysts
15. Sporozoites

S.G. Salivary glands

of pamaquin[7] and mepacrine[8], *P.cathemerium* in canaries and *P.lophurae* in chicks or ducklings have received some attention and a very useful test has been based on *P.gallinaceum* infections in chicks[9,10]. In recent years the discovery of the rodent parasite *P.berghei* has enabled experiments to be carried out in mice. For further investigation of active compounds it has been usual to work either with *P.cynomolgi*, which resembles the human parasite *P.vivax*, or with *P.knowlesi* in rhesus monkeys. Final evaluation is, of course, in man himself.

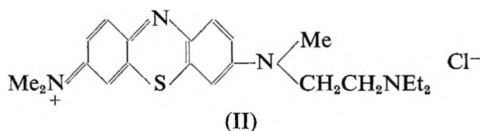
## ESTABLISHED DRUGS

The earliest antimalarial drug was quinine (I), the supremacy of which was not challenged until the second world war. In the form of cinchona bark it has been in use since the seventeenth century but it was not until 1944 that its total synthesis was reported[11].



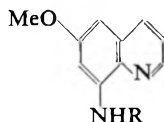

(I) Quinine

The first use of a dialkylaminoalkylamino side-chain was made by Schulemann[12] who modified the molecule of methylene blue, which had shown slight activity, to give II, which was more active than the parent




(II)

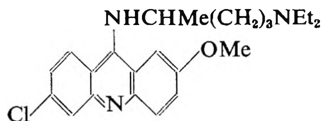
compound in avian malaria. This result led Schulemann to explore further the possibilities of the basic side-chain and its incorporation into an 8-aminoquinoline molecule provided plasmochin[13] (pamaquin, III, $R = -CH(CH_2)_3NEt_2$)




(III)

Three related compounds, pentaquin (III, $R = -(CH_2)_5NHPr^i$)[14,15], isopentaquin (III, $R = -CHMe(CH_2)_3NHPr^i$)[16,17] and primaquine (III, $R = -CHMe(CH_2)_3NH_2$) of which primaquine[18,19] seems to be the drug of choice in this series, all resulted from the American antimalarial project during the last war.

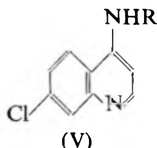
In the molecule of mepacrine (IV)[20] the basic side chain is attached to an acridine nucleus so as to form part of an extended amidine system.




(IV) Mepacrine

Chloroquine, (V, $R = -CHMe(CH_2)_3NEt_2$) which is structurally quite closely related to mepacrine, was first synthesised by Andersag[21] in 1941

but at that time it appears that the Germans preferred its 3-methyl derivative Sontochin.

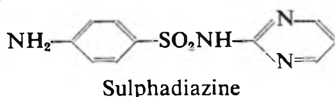



(V)

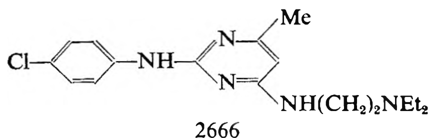
Two other 4-aminoquinolines,

amodiaquine (V, R = —$C_6H_3$($CH_2NEt_2$)—OH)[22,23] and hydroxychloroquine (V, R = —CHMe $(CH_2)_3NEtC_2H_4OH$)[24,25] have more recently become available.

When the Japanese occupied the East Indies during the second World War, supplies of quinine were denied the allied forces and an intense effort in America and Britain was made to discover a new synthetic drug. In America over 14,000 compounds were tested against avian malarias, and of these over 100 were tested in man[26]. The work in Britain led to the discovery by Curd, Davey and Rose[27,28] of proguanil (Paludrine, VI), the culmination of a sequence of steps based in the first place on pyrimidine derivatives, as outlined below (sulphadiazine was known to be active in experimental animals).




Sulphadiazine

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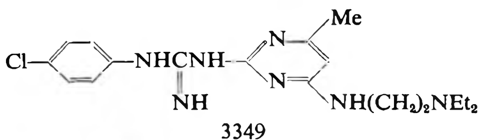


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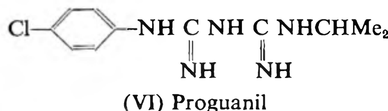
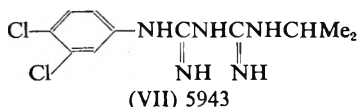



(VI) Proguanil

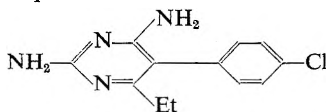
Of the many analogues of proguanil that were synthesised one deserves mention since it is now on the market (VII)[29–31].




(VII) 5943

In 1951 the pyrimidine antimalarial pyrimethamine (Daraprim, VIII) was disclosed by Russell and Hitchings[32,33]. Initial studies of 2:

4-diamino-5-aryloxypyrimidines led to the corresponding 5-aralkyl and finally to the 5-aryl compounds.



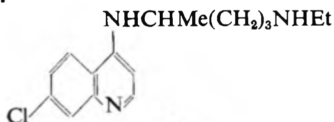

(VIII) Pyrimethamine

## Antimalarial action

Attack at more than one stage in the life cycle of the malaria parasite by drugs is possible. Quinine has a slight effect on primary gametocytes, otherwise it interferes only with the erythrocytic schizonts. Treatment of vivax or falciparum malaria with quinine reduces fever and parasitaemia, although its action, particularly against falciparum malaria, is unreliable. It cannot prevent, however, the occurrence of relapses in vivax malaria and it follows therefore that the exo-erythrocytic cycle in the liver is unaffected by it. Dosage regimes in man vary a great deal from drug to drug[34,35] and straight comparisons of recommended doses are difficult to make. For purposes of comparison, therefore, it is convenient, though not strictly applicable to human malaria, to quote minimum effective doses in a standard test system against *P.gallinaceum* in the chick. Quinine is fully active, that is parasitaemia is reduced to a level of less than about 1 per cent of infected red blood cells, compared with about 60 to 80 per cent in untreated controls, at a dose of about 2 mg./50 g. chick twice a day for $3\frac{1}{2}$ days[9,10]. At therapeutic doses in man it often causes mild side-reactions. It is implicated also in the aetiology of black-water fever. It is still used in cases of cerebral malaria.

Mepacrine, chloroquine and the other 4-aminoquinolines are similar in range of action to quinine—they are suppressive drugs having little or no effect on relapse rates of vivax malaria, and they produce radical cures of falciparum malaria because of the absence of late exo-erythrocytic forms with this infection. In the laboratory mepacrine is active at 2 mg. It was available in time to help the allied forces in malarious areas during the last war. Its concentration in the skin where its bright yellow colour often makes its presence conspicuous is a disadvantage. Chloroquine is colourless, much more active than quinine or mepacrine (it is fully active at 0·25 mg.) and rarely gives rise to toxic effects at therapeutic doses. Its action in the treatment of the overt attack is rapid and for this usage it is commonly regarded as the drug of choice. It is widely used as a suppressive, for, although not effective against early or late exo-erythrocytic forms, parasitaemia is suppressed before merozoites emerging from the liver can establish schizogony in the blood stream.

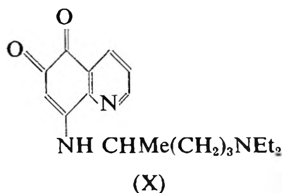
In the body chloroquine is partly converted to the compound IX[36] by loss of an ethyl group.




(IX)

The significance of the role that this compound plays in the action of chloroquine has not been elucidated. In avian malaria its activity is about the same as that of chloroquine[37] but in man when dosed orally it is poorly absorbed and is eliminated largely unchanged[36].

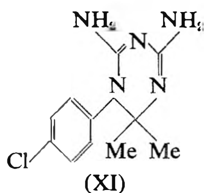
The 8-aminoquinolines are potent against erythrocytic forms in birds, for example primaquine is active at 0·02 mg. In man they were disappointing in their effect on the asexual forms in the blood and are therefore not used in treatment of attacks of malaria. They are active against both early and late exo-erythrocytic forms. Their toxicity, however precludes their routine use in causal prophylaxis but because their action against late exo-erythrocytic forms is unique they provide the only basis, in conjunction with a suppressive drug, of radical cure of vivax malaria. This series, in which primaquine with the highest therapeutic index is the drug of choice, is the only one that has a direct action on all stages of the gametocytes. Pamaquin has been shown to be metabolised to the corresponding 5:6-quinone (X)[38], which, however, is no more active *in vivo* than the parent compound.




(X)

Proguanil, 5943 and pyrimethamine are closely alike in their actions on the various stages of the parasites; and what is written about one can be taken as applicable to the others. In chicks proguanil has a marked effect on early exo-erythrocytic forms but in man whilst its action on the early tissue forms is complete with *P.falciparum* it is not so satisfactory with *P.vivax*. It is highly active against asexual blood forms (0·25 mg.) (pyrimethamine is active at 1/60th of this dose), and could therefore be classed as a suppressive—partial causal prophylactic drug. It is rather too slow in action against schizonts in man to be recommended for treatment of the overt attack. It has an indirect action on gametocytes which although they appear to be unaffected in man are prevented from completing their part of the cycle in the mosquito and the disease-transmission chain is therefore broken. These drugs therefore are of value from the public health point of view. The main advantages of this group are their effectiveness in suppression, their lack of toxicity and their cheapness.

Proguanil is without action on plasmodia growing in tissue culture but blood from animals previously dosed with the drug is markedly effective under similar circumstances[39]. The search for a metabolite the presence of which would provide a ready explanation of these results was successful. The metabolite turned out to be a dihydrotriazine derivative (XI)[40–43].

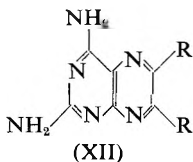


(XI)

There is no reason to doubt that it is formed by oxidation at the secondary carbon atom of the *iso*propyl group of the proguanil molecule, followed by ring-closure. The metabolite, effective at 0·025 mg., is about 10 times as active as the parent drug[41] against *P.gallinaceum* in chicks but against other experimental infections this ratio appears to be lower[44–47]. Of a large number of similar compounds synthesised[48], the most active, the corresponding 3:4-dichloro derivative (0·0025 mg.) which has been shown to be the metabolite of the corresponding diguanide 5943[41], and the proguanil metabolite have been tested clinically but although daily doses were adequate, weekly doses did not provide complete protection against *P.falciparum*[49]. In a small trial against vivax and falciparum malarias the *p*-bromo analogue also was shown to be effective[50].

## Modes of action

The structure of the dihydrotriazine metabolite is of interest in relation to the structure of pyrimethamine (VIII) which has nothing more in common with that of pteroylglutamic acid than a 2-aminopyrimidine moiety. Nevertheless the two antimalarials are intimately concerned in the utilisation by plasmodia of this growth factor. Strains of plasmodia which have been made or have become resistant to pyrimethamine are in general resistant also to proguanil[51] and *vice versa*[52]. Cross resistance to proguanil and sulphadiazine[53] and to pteridine antagonists of pteroylglutamic acid[54] of the type XII also occurs.

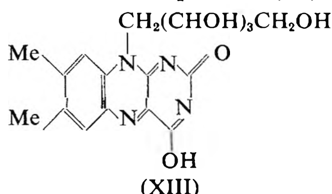


(XII)

The actions of these four types of drug against *P. gallinaceum* in chicks are all reversed by pteroylglutamic acid[55–59]. Proguanil originally said to retain its activity against *P.gallinaceum* in the presence of *p*-aminobenzoic acid[59] was later found to be antagonised by the growth factor[57,60]. A further indication of similarity of action is provided by the potentiation of the antimalarial activities of proguanil and pyrimethamine by sulphadiazine[56,61]. The antibacterial activity of the dihydrotriazines against a strain of *Streptococcus faecalis in vitro* is reversed by dihydro-, $N^{10}$-formyl-, and formyletetrahydro- pteroylglutamic acid (citrovorum factor) and by thymine but not by pteroylglutamic acid itself[62]. In this system, it is suggested therefore, that there is interference with the conversion of pteroylglutamic acid to citrovorum factor. The conclusion must be

drawn, although the situation is not free from contradictions (the action of proguanil against *P.berghei* has been said not to be inhibited by pteroylglutamic acid[60]), that pyrimethamine and the metabolite of proguanil interfere at an early stage with the synthesis by plasmodia of nucleic acids wherein single carbon units are incorporated into pyrimidine bases.

The details of the modes of action of other antimalarials are more obscure[63] even though a considerable amount of work which has been reviewed by Fulton[63], has been done on the effects of quinine and mepacrine in particular on the metabolism of the malaria parasite. The relationship in structure between mepacrine (IV) and riboflavine (XIII)



(XIII)

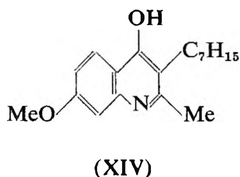
is fairly obvious and efforts to demonstrate antagonism between the two have been made[64]. The vitamin does indeed reverse the action of mepacrine, and of quinine, pamaquin and chloroquine, against *Lactobacillus casei*[65]. The effect of adenosine in producing a heart block in guinea pigs is reversed by all the drugs just mentioned[66]. The significance of these results in relation to antimalarial action has not been demonstrated.

Much has been written on the constitution of antimalarial drugs in relation to their biological activity. The basic side-chain and the heterocyclic nucleus both common to so many active compounds have often been referred to respectively as the part of the molecule necessary to bring the drug into action at the required site within the host, and the toxophoric centre[67,68]. The hypothesis that a tautomeric system as in mepacrine and the 4-aminoquinolines[69] was essential for activity, might be stretched to cover the activity of the 8-aminoquinolines now that an active metabolite, the 5:6-quinone, is known which should be capable of undergoing tautomerisation to a 6-hydroxy-5:8-quinoneimine. This sort of speculation is necessarily limited in application and can hardly be expected to lead to a true understanding of the way these drugs act.

## Other active compounds

Of the drugs that have during or since the last war been extensively tested and finally rejected or are still awaiting a final assessment the following are selected for brief mention.

Endochin (XIV)[70] the best of over 100 compounds related to it has some effect on early exo-erythrocytic forms in bird malaria but has no effect in man.



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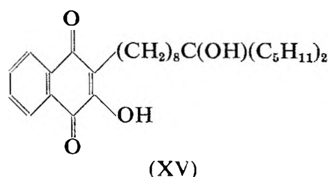


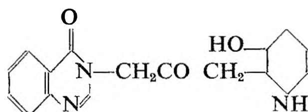
(XV)

Lapinone (XV) a member of a long series of naphthoquinone derivatives prone to degradation in the human body has been stated to possess definite action in vivax malaria after intravenous injection[71,73].

Ch'ang Shan (XVI)[74–78] shown to be identical with an alkaloid from the hydrangea[79,80] is very active in laboratory animals but low activity in man coupled with a powerful emetic action[81,82] has made the type unacceptable.

A small number of 3-amino-1:2:4-benztriazines have shown an interesting level of activity in the laboratory[83].

Many Mannich bases derived mostly from phenols have been examined[84] but only incorporation into a 4-aminoquinoline as in amodiaquine[22] or in the recently tested corresponding pyrrolidyl compound Propoquine[85] gives this type sufficient activity to be of practical importance. More recently however, the series has been extended and the compound XVII[86] has proceeded to clinical trial. It proves to be about one-third to one-half as active as chloroquine in the treatment of falciparum malaria[87].

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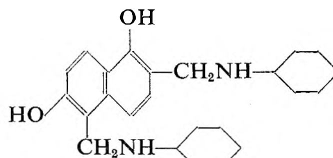


(XVII)

Another recent clinical trial has been concerned with a diaza-anthracene analogue of mepacrine, azacrine[88], which is comparable in activity with chloroquine against *P.falciparum* but slower in action against *P.-malariae*[89–91].

With the exception of the naphthoquinones all the compounds in this list are bases. Beyond this it is clearly impossible to generalise.

In conclusion it can be said that the battery of drugs for the treatment and prevention of malaria is almost as complete as possible. There are available for the treatment of the overt attack and for the radical cure of falciparum malaria, chloroquine or some other 4-aminoquinoline; for suppression, proguanil, pyrimethamine or chloroquine; for radical cure of vivax malaria, primaquine or some other 8-aminoquinoline, and for interruption of the transmission of the disease there are proguanil and pyrimethamine.

To emphasise the point it has recently been written that freedom from malaria can be practically guaranteed for any intelligent person and his family at a cost not exceeding the price of 100 cigarettes per annum[92].

## References

1. Bray, *Amer. J. trop. Med. Hyg.*, 1957, **6**, 514.
2. Shortt, Garnham, Covell and Shute, *Brit. med. J.*, 1948, **1**, 547.
3. Shortt, Fairley, Covell, Shute and Garnham, *ibid.*, 1949, **2**, 1006.
4. Bray, *Trans. Roy. Soc. trop. Med. Hyg.*, 1957, **51**, 248.
5. Shortt and Garnham, *Brit. med. J.*, 1948, **1**, 1225.
6. Shortt, *Brit. med. Bull.*, 1951, **8**, 7.
7. Roche, *Arch. Schiffs-u. Tropenhyg.*, 1926, **30**, 11.
8. Kikuth, *Dtsch. med. Wschr.*, 1932, **58**, 530.

9. Davey, *Ann. trop. Med. Parasit.*, 1946, **40**, 52.
10. Davey, *ibid.*, 1946, **40**, 453.
11. Woodward and Doering, *J. Amer. chem. Soc.*, 1944, **66**, 849.
12. Schulemann, *Proc. Roy. Soc. Med.*, 1932, **25**, 897.
13. Schulemann, Schönhöfer and Wingler, *Klin. Wschr.*, 1932, **11**, 381.
14. Drake and Van Hook, *Brit. Pat.*, 637, 863.
15. Loeb, *J. Amer. med. Ass.*, 1946, **132**, 321.
16. Elderfield and Head, *Sw. Pat.*, 270, 396.
17. Alving, *Proc. 4th Intern. Congr. trop. Med. Mal., Wash.*, 1948, **1**, 734.
18. Elderfield, *Brit. Pat.*, 695, 159, *J. Amer. chem. Soc.*, 1955, **77**, 4816.
19. Edgcomb, Arnold, Yount, Alving, Eicheberger, Jeffrey, Eyles and Young, *J. Nat. mal. Soc.*, 1950, **9**, 285.
20. Mauss and Mietszch, *Klin. Wschr.*, 1933, **12**, 1276.
21. Andersag, Breitner and Jung, *Ger. Pat.*, 683, 692.
22. Burckhalter, Tendick, Jones, Jones, Holcomb and Rawlins, *J. Amer. chem. Soc.*, 1948, **70**, 1363.
23. Payne, Sharp and Nickel, *Amer. J. trop. Med.*, 1949, **29**, 353.
24. Surrey and Hammer, *J. Amer. chem. Soc.*, 1950, **72**, 1814.
25. Loughlin, Rice, Wells, Rappaport and Joseph, *Antibiot. Chemother.*, 1952, **2**, 171.
26. *Survey of Antimalarial Drugs*, F. Y. Wiselogle, Editor, J. W. Edwards, Ann Arbor, Michigan, 1946.
27. Curd and Rose, *J. chem. Soc.*, 1946, 729.
28. Curd, Davey and Rose, *Ann. trop. Med. Parasit.*, 1945, **39**, 208.
29. Curd, Davey, Hendry and Rose, *Brit. J. Pharmacol.*, 1950, **5**, 438.
30. Crowther, Curd, Davey, Hepworth, Hendry and Rose, *J. chem. Soc.*, 1951, 1774.
31. Robertson, *Trans. Roy. Soc. trop. Med. Hyg.*, 1957, **51**, 457.
32. Russell and Hitchings, *J. Amer. chem. Soc.*, 1951, **73**, 3763.
33. Falco, Goodwin, Hitchings, Rollo and Russell, *Brit. J. Pharmacol.*, 1951, **6**, 185.
34. Davey, *Brit. med. Bull.*, 1951, **8**, 42.
35. Boyd, *Brit. med. J.*, 1954, **2**, 148.
36. Titus, Craig, Golumbic, Mighton, Wempen, and Elderfield, *J. org. Chem.*, 1948, **13**, 39.
37. *Survey of Antimalarial Drugs*, Vol. II, F. Y. Wiselogle, Editor, J. W. Edwards, Ann Arbor, Michigan, 1946, p. 1142.
38. Josephson, Greenberg, Taylor and Bami, *J. Pharmacol.*, 1951, **103**, 7.
39. Hawking and Perry, *Brit. J. Pharmacol.*, 1948, **3**, 320.
40. Carrington, Crowther, Davey, Rose and Levi, *Nature, Lond.*, 1951, **168**, 1080.
41. Crowther and Levi, *Brit. J. Pharmacol.*, 1953, **8**, 93.
42. Carrington, Crowther and Stacey, *J. chem. Soc.*, 1954, 1017.
43. Bailey, *Acta. Cryst.*, 1954, **7**, 366.
44. Schmidt, Loo, Fradkin and Hughes, *Proc. Soc. exp. Biol. N.Y.*, 1952, **80**, 367.
45. Modest, Foley, Pechet and Farber, *J. Amer. chem. Soc.*, 1952, **74**, 855.
46. Nair, Bami and Ray, *Ind. J. Mal.*, 1955, **9**, 105.
47. Krishnaswami, Prakash, Bami, Ramakrishnan, *ibid.*, 1953, **7**, 229 (*Trop. Dis. Bull.*, 1954, **51**, 773).
48. Crowther, *Brit. Pat.*, 709, 906.
49. Robertson, *Trans. Roy. Soc. trop. Med. Hyg.*, 1957, **51**, 488.
50. Ray, Bami and Basu, *J. Ind. med. Ass.*, 1956, **26**, 217. (*Trop. Dis. Bull.*, 1956, **53**, 973).
51. Rollo, *Nature, Lond.*, 1951, **168**, 332.
52. Robertson, Davey and Fairley, *Brit. med. J.*, 1952, **2**, 1255.
53. Bishop and McConnachie, *Parasitol.*, 1950, **40**, 163.
54. Greenberg, *J. Pharmacol.*, 1949, 97, 484.
55. Greenberg, *Proc. Soc. exp. Biol. N.Y.*, 1949, **71**, 306.
56. Rollo, *Brit. J. Pharmacol.*, 1955, **10**, 208.
57. Greenberg, *Exp. Parasit.*, 1953, **2**, 271.
58. Greenberg and Richeson, *Proc. Soc. exp. Biol. N.Y.*, 1951, **77**, 174.
59. Bishop and McConnachie, *Nature, Lond.*, 1948, **162**, 541.
60. Thurston, *Lancet*, 1950, **259**, 438.
61. Greenberg, Boyd and Josephson, *J. Pharmacol.*, 1948, **94**, 60.
62. Foley, *Proc. Soc. exp. Biol. N.Y.*, 1953, **83**, 733 *et seq.*
63. Fulton, *Brit. med. Bull.*, 1951, **8**, 26.
64. Silverman and Evans, *J. biol. Chem.*, 1944, **154**, 521.
65. Madinaveitia, *Biochem. J.*, 1946, **40**, 373.
66. Madinaveitia and Raventos, *Brit. J. Pharmacol.*, 1949, **4**, 81.
67. Magidson, Delektorskaya, Lipowitsch, *Arch. Pharm.*, 1934, **272**, 74.

68. Magidson and Grigorowski, *Ber.*, 1936, **69**, 396.
69. Schönhöfer, *Z. physiol. Chem.*, 1942, **274**, 1.
70. Curtis, Davis, Smadel, Southworth and Volwiler, *C.I.O.S. Report XXIII*—12, pp. 24, 31.
71. Fieser, Leffler and others, *J. Amer. chem. Soc.*, 1948, **70**, 3206.
72. Fawaz and Fieser, *ibid.*, 1950, **72**, 996.
73. Fawaz and Haddad, *Amer. J. trop. Med.*, 1951, **31**, 569.
74. Jang, Fu, Wang, Huang, Lu and Chou, *Science*, 1946, **103**, 59.
75. Koepfli, Mead and Brockman, *J. Amer. chem. Soc.*, 1947, **69**, 1837.
76. Henderson, Rose, Harris and Chen, *J. Pharmacol.*, 1949, **95**, 191.
77. Koepfli, Mead and Brockman, *J. Amer. chem. Soc.*, 1949, **71**, 1048.
78. Koepfli, Brockman and Moffat, *ibid.*, 1950, **72**, 3323.
79. Ablondi, Gordon, Morton and Williams, *J. org. Chem.*, 1952, **17**, 14 *et seq.*
80. Hewitt, Wallace, Gill and Williams, *Amer. J. trop. Med. Hyg.*, 1952, **1**, 768.
81. Trevino, Reyes and Mendoza, *Rev. Inst. Sal. Enferm. Trop.*, 1953, **13**, 253. (*Trop. Dis. Bull.*, 1954, **51**, 464.)
82. Edeson and Wilson, *Trans. Roy. Soc. trop. Med. Hyg.*, 1955, **49**, 543.
83. Wolf, Pfister, Wilson and Robinson, *J. Amer. chem. Soc.*, 1954, **76**, 3551.
84. Burckhalter, Tendick, Jones, Holcomb and Rawlins, *ibid.*, 1946, **68**, 1894.
85. Hoekenga, *Amer. J. trop. Med. Hyg.*, 1957, **6**, 987.
86. Duffin and Rollo, *Brit. J. Pharmacol.*, 1957, **12**, 171.
87. Bruce-Chwatt and Charles, *Brit. med. J.*, 1957, **2**, 23.
88. Golberg and Besly, *Brit. Pat.*, 722, 704.
89. Bruce-Chwatt and Archibald, *Brit. med. J.*, 1953, **1**, 539.
90. Ang'awa and Fendall, *J. trop. Med. Hyg.*, 1954, **57**, 59.
91. Edeson, *Ann. trop. Med. Parasit.*, 1954, **48**, 160.
92. Wilson and Edeson, *Med. J. Malaya*, 1957, **11**, 190. (*Trop. Dis. Bull.*, 1957, **54**, 1155.)

# RESEARCH PAPERS

## THE PHARMACOLOGY OF PROPIONYL ATROPINE METHYL NITRATE

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A series of esters of atropine and hyoscine, both in the tertiary and quaternary form, have been examined for their muscarinic and ganglionic blocking and neuromuscular transmission inhibiting activity. In addition the effect on the gastric secretion has been studied by the Shay rat method. Figures are also given for toxicities. In general, the pharmacology of the quaternary derivatives is qualitatively and quantitatively the same as the parent substances. The quaternary compounds retain a considerable amount of their tertiary activity but in addition become relatively strong ganglionic blocking agents. Most of the compounds reduce the volume and acidity of the gastric juice.

In recent years several new compounds have been introduced into medicine for the symptomatic relief of peptic ulcers. Most are quaternary ammonium compounds differing pharmacologically from the natural and synthetic tertiary amines in producing their effects by ganglionic blockade, whilst the tertiary amines block the effector organs innervated by post-ganglionic cholinergic nerves. In an earlier paper we have shown that the acylation of hyoscine produced esters whose action was prolonged but less marked than atropine on the central nervous system[1]. It therefore seemed of interest to prepare the atropine analogues and study their pharmacology. It was possible also to prepare the quaternary derivatives of these esters. While quaternisation would be expected to profoundly alter the pharmacological properties of the compounds, perhaps even to the extent of producing a curariform action[2], these quaternary compounds have been shown to retain some of their original pharmacological properties together with mild ganglionic blocking powers, the neuromuscular junction being only slightly affected.

### Methods

Materials used. Atropine sulphate, atropine methyl bromide (AMB), hyoscine hydrobromide, propionyl atropine methyl nitrate (PAMN), valeryl atropine methyl bromide (VAMB), hyoscine methyl bromide, *N*-butyryl hyoscine bromide, (Buscopan), oxyphenonium (Antrenyl), pipenzolate (Piptal), and pentamethonium (C5).

#### *Isolated Intestine*

The spasmolytic potency of the test drug compared with atropine in inhibiting acetylcholine-induced spasms of guinea pig isolated ileum was used as an index of post ganglionic cholinergic nerve blocking action. The dose of acetylcholine—0·1 ml., concentration range $10^{-6}$ to $10^{-7}$ w/v causing a sub-maximal contraction of the ileum was determined. For

each preparation the concentration of atropine and then test drug which when left in the bath for 30 seconds partially or completely inhibited for 15 to 25 minutes this acetylcholine induced contraction of the ileum was determined. All drug solutions were made up on a molecular basis.

TABLE I

ANTI-ACETYLCHOLINE ACTIVITY OF SOME ATROPINE ESTERS ON THE GUINEA PIG ILEUM

| Drug | No. of experiments averaged | Molar concentration | Inhibition Time (minutes) |
|---|---|---|---|
| Hyoscine hydrobromide .. .. | 13 | $0{\cdot}22 \times 10^{-8}$ | 12 |
| Atropine hydrobromide .. .. | 13 | $0{\cdot}5 \times 10^{-8}$ | 12 |
| Atropine methylbromide .. | 5 | $0{\cdot}6 \times 10^{-8}$ | 15 |
| Acetylatropine hydrobromide .. | 5 | $0{\cdot}7 \times 10^{-8}$ | 12 |
| Acetylatropine methylbromide | 3 | $0{\cdot}5 \times 10^{-7}$ | 16 |
| Propionylatropine hydrobromide | 5 | $0{\cdot}75 \times 10^{-8}$ | 13 |
| Propionylatropine methylbromide | 4 | $0{\cdot}2 \times 10^{-7}$ | 13 |
| Valerylatropine methylbromide | 1 | $0{\cdot}2 \times 10^{-8}$ | 15 |

Using this method each preparation acted as its own control against atropine, and so eliminated errors due to the variation in sensitivity of different preparations.

TABLE II

GANGLIONIC BLOCKING ACTIVITY OF SOME DRUGS ON THE CAT SUPERIOR CERVICAL GANGLION

| Cat. No. | Drug | Dose mg./kg. | Time in minutes | |
|---|---|---|---|---|
| | | | total block | partial block |
| 1 | PAMN | 1 | 4 | 11 |
| | Atropine sulphate | 1 | No block | 10 |
| | AMB | 1 | 5 | 6 |
| 3 | PAMN | 1 | No block | 9 |
| | Atropine sulphate | 1 | ,, | No block |
| | AMB | 1 | ,, | 8 |
| | VAMB | 1 | ,, | 4 |
| | Buscopan | 1 | ,, | 5 |
| | Hyoscine MeBr | 1·7 | ,, | No block |
| 4 | PAMN | 1·7 | 4 | 8 |
| | Atropine sulphate | 1·7 | No block | 4 |
| | Hyoscine MeBr | 0·7 | ,, | No block |
| | | 1·3 | ,, | 6 |
| | | 3·3 | ,, | 8 |
| | AMB | 0·7 | ,, | 6 |
| | | 1·7 | ,, | 13 |
| | PAMN | 0·7 | ,, | 9 |
| | | 1·7 | ,, | 15 |
| | VAMB | 0·7 | ,, | 11 |
| | | 1·7 | ,, | 15 |
| | Oxyphenonium | 1·7 | ,, | No block |
| | | 3·3 | ,, | 4 |
| | Pipenzolate | 3·3 | ,, | 5 |
| | PAMN | 1·7 | ,, | 8 |
| | | 3·3 | ,, | 13 |
| | C 5 | 3·3 | ,, | 30 |
| 6 | Hyoscine MeBr | 1·0 | ,, | 8 |
| | PAMN | 1·7 | 2 | 11 |
| | AMB | 1·7 | 1 | 10 |
| | VAMB | 1·0 | No block | 6 |
| | Pipenzolate | 1·0 | ,, | 7 |
| | Oxyphenonium | 1·0 | ,, | 7 |
| | PAMN | 1·0 | ,, | 11 |
| | AMB | 1·0 | ,, | 14 |
| | C 5 | 1·0 | ,, | 37 |

Total block means the time during which there is no contraction of the nictitating membrane, partial block means the time from the first signs of recovery until this is complete.

*Cat Superior Cervical Ganglion*

The ganglion was dissected with its accompanying pre- and post-ganglionic fibres (the sympathetic were carefully separated from the parasympathetic fibres). The drugs were injected intravenously and the contraction of the nictitating membrane was recorded after appropriate stimulation of the pre-, and post ganglionic fibres.

*Shay Rat*

The operative technique of Shay and others[3,4] was followed. The rats were anaesthetised with 20 mg./kg. of pentobarbitone i.p. As gastric secretion does not take place whilst the animal is unconscious, 20 mg./kg.

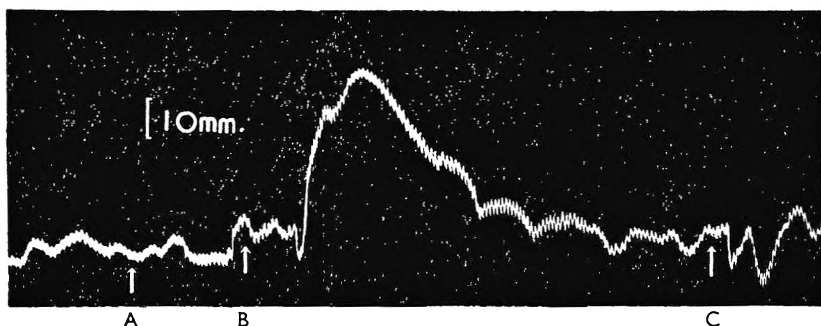


FIG. 1. A, 12.30 p.m. PAMN 0·17 mg./kg; B, 1.10 p.m. nicotine 0·2 mg./kg.; C, 1.33 p.m. PAMN × nicotine 0·17 and 0·2 mg./kg.

of bemegride was injected i.p. at the end of the operation and caused an almost immediate arousal of the animals. The drugs used were injected subcutaneously, immediately at the completion of the operation.

*Neuromuscular Junction*

The neuromuscular blocking action of PAMN was tested on the rat phrenic nerve-diaphragm preparation[5].

*Toxicity*

The acute toxicity was estimated on mice by the intraperitoneal route and the LD50 was calculated by the standard probit technique. Semi-acute toxicity tests were carried out on mice and rats by injecting the animals intraperitoneally twice a day for five days at a dose ratio of 0·56 of the LD50 to mice.

## RESULTS

*Isolated Intestine*

The anticholinergic activity of the atropine derivatives is given in Table I. The results indicate that the degree of activity of hyoscine is about twice that of atropine and that esterification does not affect the activity quantitatively or qualitatively. However quaternisation of the esters does result in a decrease in spasmolytic activity.

*Superior Cervical Ganglion*

The ganglionic blocking activity of the atropine derivatives is shown in Table II. The order is hyoscine methyl bromide < *N*-butyryl hyoscine bromide, oxyphenonium, pipenzolate < AMB, PAMN < VAMB, < C5. Thus PAMN has marked ganglionic blocking activity but is not as

TABLE III

THE EFFECT OF DRUGS ON BLOOD PRESSURE OF THE ANAESTHESTISED CAT

| Drug | Dose mg./kg. | Fall mm.Hg | Duration minutes |
|---|---|---|---|
| Hyoscine MeBr .. | 1 | 33 | 4 |
| PAMN .. .. | 1·7 | 48 | 9 |
| AMB .. .. | 1·7 | 41 | 11 |
| VAMB .. .. | 1 | 35 | 10 |
| Pipenzolate.. .. | 1 | 23 | 5 |
| Oxyphenonium .. | 1 | 15 | 5 |
| PAMN .. .. | 1 | 35 | 10 |
| AMB .. .. | 1 | 37 | 8 |
| C 5 .. .. | 1 | 36 | 45 |
| Hyoscine MeBr .. | 1 | 33 | 4 |

powerful as pentamethonium. The site of action of PAMN is at the ganglion because stimulation of the post ganglionic fibres, when the ganglion is blocked, still results in contraction of the nictitating membrane.

*PAMN and Nicotine.* As is well known nicotine stimulates all sympathetic ganglia, producing contraction of the nictitating membrane and a rise in the blood pressure.

TABLE IV

SHAY RAT EXPERIMENT

| Drug dose, 350 mg./kg. | Number experiments averaged | Collection time hours | Analysis of Stomach Content | | |
|---|---|---|---|---|---|
| | | | total volume | pH | m.eq. of acid per kg. |
| Atropine hydrobromide .. .. .. | 17 | 22 | 1·2 | 3·9 | 0·329 |
| Atropine methyl bromide .. .. | 4 | 20 | 1·7 | 3·8 | 0·420 |
| Methyl atropine methyl bromide .. | 1 | 22 | ? | ? | 0·139 |
| Acetyl atropine hydrobromide .. .. | 4 | 21 | 0·95 | 4·0 | 0·480 |
| Acetyl atropine methyl bromide .. | 4 | 20 | 0·48 | 5·2 | 0·277 |
| Propionyl atropine methyl bromide .. | 7 | 22 | 1·54 | 5·8 | 0·227 |
| Propionyl atropine methyl nitrate .. | 8 | 22 | 1·4 | 4·4 | 0·206 |
| Valeryl atropine methyl nitrate .. .. | 9 | 21 | 1·2 | 3·3 | 0·471 |
| Hyoscine hydrobromide .. .. .. | 7 | 21 | 2·2 | 2·9 | 1·201 |
| Acetyl hyoscine hydrobromide .. .. | 3 | 20 | 1·95 | 3·6 | 0·368 |
| Hyoscine methyl bromide .. .. | 6 | 21 | 1·50 | 4·6 | 0·508 |
| Propionyl hyoscine hydrobromide .. | 7 | 21 | 1·84 | 3·1 | 0·981 |
| Butyryl hyoscine hydrobromide .. | 7 | 22 | 2·3 | 3·5 | 0·662 |
| Valeryl hyoscine hydrobromide .. .. | 3 | 21 | 1·17 | 4·1 | 0·398 |
| *iso*Valeryl hyoscine hydrobromide .. | 9 | 21 | 2·0 | 3·5 | 0·844 |
| Benzoyl hyoscine hydrobromide (250 mg./kg.) .. .. .. .. | 4 | 21 | 1·6 | 5·6 | 0·507 |
| Benzoyl hyoscine hydrobromide (220 mg./kg.) .. .. .. .. | 1 | 21 | 4·2 | 3·0 | 2·579 |
| Benzoyl hyoscine hydrobromide (157 mg./kg.) .. .. .. .. | 1 | 21 | 1·7 | 3·5 | 0·686 |
| Acetyl hyoscine amino oxide hydrobromide | 2 | 21 | 2·7 | 2·5 | 0·620 |
| Acetyl hydrocyamine hydrobromide .. | 2 | 21 | 3·3 | 2·5 | 0·852 |
| Acetyl homatropine hydrobromide .. | 2 | 22 | 2·6 | 2·3 | 0·950 |
| Benzoyl homatropine hydrobromide (200 mg./kg.) .. .. .. .. | 2 | 22 | 0·7 | 5·8 | 0·084 |
| No Drug .. .. .. .. .. | 3 | 22 | 9·7 | <2·0 | 2·541 |

If the PAMN and nicotine were injected simultaneously the results were variable; the usual pattern was a rise in blood pressure and contraction of the nictitating membrane less than that given by the control dose of

nicotine. The control doses of PAMN had little effect on the blood pressure and no effect on the nictitating membrane. One interpretation of these results would be that the PAMN was antagonising the effect of nicotine at the ganglia (see Fig. 1). When PAMN itself produced a fall

TABLE V

SUMMARY—SHAY-RAT EXPERIMENT

Drugs which produced a pH of 6 or more in rat stomach.

| Drug | Number of experiments | pH 6 or more |
|---|---|---|
| Atropine hydrobromide .. .. | 17 | 2 (pH 7·3, pH 8·0) |
| Acetyl atropine methyl bromide .. | 4 | 1 (pH 8·5) |
| Propionyl atropine methyl bromide .. | 7 | 3 (pH 8·5, pH 7·0, pH 7·5) |
| Propionyl atropine methyl nitrate .. | 8 | 1 (pH 8·8) |
| Hyoscine methyl bromide .. .. | 6 | 1 (pH 7·9) |
| *iso*Valeryl hyoscine hydrobromide .. | 9 | 1 (pH 6·4) |
| No Drug (Control) .. .. .. | 3 | 0 (pH $< 2$ in all cases) |

in blood pressure of any magnitude it could be argued that the anullment of the blood pressure rise due to the nicotine plus PAMN was the result of the summing of the effects peripherally.

*Effect on Blood Pressure*

All the compounds tested gave a fall in blood pressure (Table III). In general the results on the blood pressure paralleled the ganglionic blocking activity of the compounds.

TABLE VI

THE EFFECT OF SOME ATROPINE ESTERS ON THE RAT PHRENIC NERVE DIAPHRAGM

| Drug | Molar concentration in bath | Time for complete inhibition (minutes) |
|---|---|---|
| Tubocurarine | $1 \times 10^{-6}$ | 5 |
| ,, | $1 \times 10^{-5}$ | 1·5 |
| Atropine .. | $1 \times 10^{-3}$ | No effect |
| AMB .. | $1 \times 10^{-3}$ | 3 |
| ,, .. | $5 \times 10^{-4}$ | 5 |
| PAMN .. | $1 \times 10^{-3}$ | 2 |
| ,, .. | $5 \times 10^{-4}$ | 5 |
| ,, .. | $2 \times 10^{-4}$ | No effect |
| VAMB .. | $5 \times 10^{-4}$ | 1 |
| ,, .. | $5 \times 10^{-5}$ | Slight effect |

*Shay Rat*

As the compounds so far tested had both anti-muscarinic and ganglionic blocking activity it appeared as though they might be useful for the symptomatic treatment of peptic ulcers. The results are shown in Tables IV and V.

This test illustrated that a single subcutaneous dose of the drugs decreased the total volume and the acidity of gastric secretion (for a period of 18 to 22 hours). It has been shown (Shay and others[3,4]), that if unbuffered gastric juice is allowed to accumulate in the stomach of the rat for this period, ulcerations of the stomach are produced and the stomach is distended with highly acid gastric fluid, pH below 2. Both

these symptoms were reduced by atropine, hyoscine, or any of the derivatives tested. No ulceration occurred in any medicated animal, and the volume of secretion was also greatly reduced. Because of wide individual variation between animals, 121 rat experiments were performed. The results indicate that the atropine compounds exhibit a stronger antisecretory activity than the hyoscine compounds. In each case where

TABLE VII

TOXICITY (LD 50 MICE)*

| Substance | mg./kg. intraperitoneal | Limits 0·95 | mg./kg. Oral |
|---|---|---|---|
| Propionyl atropine methyl nitrate | 114 | 93–141 | 2000 |
| Benzoyl hyoscine hydrobromide.. | 158 | 144–172 | |
| Atropine sulphate .. .. | 355 | 329–384 | 1000 |
| Hyoscine hydrobromide .. .. | 757 | 732–783 | |

* The LD 50 for the substances by the intraperitoneal route is calculated by means of probit analysis. The figures for the oral route are only an approximation because it was evident that the compounds were non-toxic and to have completed a statistical series would have required more material than was available.

tertiary and quaternary compounds were investigated, the quaternary derivatives were considerably more active. Several of the compounds caused an alkaline gastric content in some of the test animals. (See Table IV.) In these instances the total volume of secretion although small, was not so small as to be entirely attributable to swallowed saliva, particularly as these drugs tend to decrease salivary secretion. Normal gastric secretion is a mixture obtained from three types of secretory cells, which may not be equally affected by these drugs. Probably a greater inhibition of the acid-secreting cells was obtained than of the mucin-secreting cells. The secretion of the latter is alkaline in nature and also because of its high protein content acts as a buffer for any small amount of acid that may be present.

TABLE VIII

TOXICITY (SEMI-ACUTE)

| Substance | Dose mg./kg. i.p. | Injection frequency | No. of animals | Period to death |
|---|---|---|---|---|
| Propionyl atropine methyl nitrate .. | 64·25 | 2 per day | 10 rats | did not die within 5 days |
| ,, ,, ,, ,, .. | ,, | ,, | 10 mice | 3 died 4–5 days |
| Atropine sulphate .. .. .. | 200 | ,, | 6 rats | 2 died 3–4 days |
| | ,, | ,, | 6 mice | 3 died 3–5 days |

*Neuromuscular Junction*

PAMN has a curariform action at the neuromuscular junction. This action is only one fiftieth that of tubocurarine. Other results are given in Tabel VI.

*Toxicity*

Table VII gives the intraperitoneal LD 50 to mice of several of these compounds. Table VIII gives the semi-acute toxicity when the drugs were administered at a ratio of 0·56 of their LD 50 to mice. During the Shay Rat experiments it was noted that benzoyl hyoscine hydrobromide

was very toxic. The dose used for other compounds 350 mg./kg.,resulted in the death of all animals. As has been found elsewhere the toxicity of quaternary compounds is much less by mouth than by parenteral administration[2,6].

## Discussion

In an earlier paper Shaw and Rosenblum[1] postulated that the esters (acetyl, butyryl, propionyl etc.) of hyoscine might owe their lesser degree of activity but more prolonged action to the fact that they were hydrolysed in the body before they exerted their pharmacological actions. Subsequent unpublished work has not confirmed this. In the present series the acetyl and propionyl esters of atropine had the same qualitative and quantitative spasmolytic action on the guinea pig ileum. Quaternisation reduced the anti-muscarinic activity to one tenth that of the tertiary amines.

As was to be expected the quaternary derivatives showed ganglionic blocking activity. The activity of PAMN was about the same as that of atropine methyl bromide but greater than that of the other therapeutically employed gastric "sedatives".

PAMN also caused a fall in blood pressure in the anaesthetised cat, presumably due to its ganglionic blocking activity. A fall in blood pressure was not noted in 6 paraplegic patients receiving 24 mg. of PAMN per day. Peptic ulcer patients receiving the drug for a period of 6 months, at about the same dose level had no complaints of orthostatic hypotension.

It was not surprising that several of the atropine and hyoscine derivatives reduced the volume and acidity of the gastric content of the Shay rat preparation. It is interesting that the quaternary derivatives were as active, or more active, than atropine. Yet these compounds had only one tenth the antiacetylcholine activity on the guinea pig ileum. It would thus appear that ganglionic block is more important than anti-muscarinic activity in the prevention of gastric secretion.

The results of toxicity tests of PAMN and related compounds at first appear to be paradoxical. By the intraperitoneal route PAMN was three times as toxic as atropine, and benzoyl hyoscine hydrobromide was six times as toxic as hyoscine. Orally PAMN was only half as toxic as atropine. These findings are in agreement with work of other authors[2,6], who have shown that quarternary are more toxic than tertiary compounds when given parenterally and the reverse holds orally. We have shown the same concept to be true for quinine (Shaw and others unpublished) When a fixed ratio of the LD 50 was chosen for a semi-acute toxicity the quaternary compound appeared to be relatively less toxic.

PAMN had slight neuromuscular junction blocking activity but no muscular weakness was noticed in man consuming this drug over long periods.

PAMN thus shows possibilities as a therapeutic substance for symptomatic treatment of peptic ulcer. This matter will be considered further in another publication (Herman and Shaw[7]).

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## References

1. Shaw and Rosenblum, *Austral. J. Sci.*, 1954 **17**, 106.
2. Brown and Fraser, *Trans. Roy. Soc. Edinb.*, 1869, **25**, 693.
3. Shay, Sun and Gruenstein, *Gastroenterology*, 1945, **5**, 43.
4. Shay, Komarov, Fels, Meranze, Gruenstein and Siplet, *ibid.*, 1954, **26**, 906.
5. Bülbring, *Brit. J. Pharmacol.*, 1946, **1**, 38.
6. Shea, *ibid.*, 1956, **11**, 171.
7. Herman and Shaw, *J. Pharm. Pharmacol.*, 1958, **10**, 356.

# THE ACTION OF PROPIONYL ATROPINE METHYL NITRATE ON GASTRIC FUNCTION

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A quaternary compound, propionyl atropine methyl nitrate, previously demonstrated to have muscarinic and ganglionic blocking properties has been shown to decrease the volume and acidity of the gastric juice in man. Using a variation of the glucose tolerance test the compound was also shown to reduce the motility of the alimentary tract. Side effects due to parasympathetic blockade were in most cases absent at the doses used. A clinical trial with 26 cases of peptic ulcer has resulted in a complete alleviation of symptoms without the production of side actions. In six cases the trial has extended for six months without the development of tolerance.

In a previous paper[1] several quaternary ammonium esters of atropine, were shown to possess both muscarinic and ganglionic blocking activity. This combination of these properties in one molecule together with the capacity to inhibit gastric secretion, in the Shay Rat preparation suggested their use for the symptomatic treatment of peptic ulcers. This paper attempts an investigation of the effect of one of these esters on the gastric secretion and gastric motility in man. The results were compared with those obtained with atropine, atropine methyl bromide and hyoscine methyl bromide. The trial substance propionyl atropine methyl nitrate (PAMN) checked both secretion and motility with an almost complete absence of side actions.

Screening a potential drug for the treatment of peptic ulcer is fraught with difficulties. This applies both to the measurement of the secreted acid and the motility of the alimentary canal. The problem is discussed by Bachrach and others[2]. In view of the difficulties we decided on simplicity of method. The histamine-stimulated secretion test was rejected as this procedure could give erroneous results, since the physical discomfort produced by histamine or the apprehension of an injection in untrained volunteers could result in a variable inhibition of gastric secretion. We therefore decided to use the alcohol test meal.

The direct measurement of motility is even more unsatisfactory, especially without the services of a gastroenterological unit. The dependence on volunteers makes it necessary to employ an indirect method which will cause little or no discomfort. Indirect methods are discussed by Bachrach and others but as none appeared to offer any advantages the following method was devised.

On the assumption that the stomach itself absorbs little or no sugar the initial rise of blood sugar after the ingestion of a large amount of glucose or sucrose depends upon the ingested sugars reaching the duodenum. Any lack of motility of the stomach would thus delay the rate of rise of blood sugar. Similarly a lessening of intestinal motility would be expected to enhance this effect. A flattening of the blood sugar curve would

also be expected since the slower rate of absorption would permit of a more effective control by the insulin secretory mechanism of the endocrine system.

To demonstrate that the slower rate of sucrose absorption was not solely due to a decrease in acid hydrolysis, or to a decrease in the enzymatic splitting activity of the gastric mucosa, both glucose and sucrose tests were made. The results were more consistent than might be expected and we find that the glucose tolerance test could be extended to yield evidence of alimentary movement with a minimum of discomfort to the subject.

TABLE I

THE pH OF INDIVIDUAL GASTRIC SAMPLES AFTER SINGLE ORAL DOSES

Subject I.

| Time minutes | Control no drug | Atropine 1·3 mg. | Propionyl atropine methyl nitrate | | | | Valeryl atropine methyl bromide | | Atropine methyl bromide 8 mg. | Hyoscine methyl bromide 8 mg. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 2 mg. | 4 mg. | 6 mg. | 8 mg. | 5 mg. | 8 mg. | | |
| Fasting | 1·47 | 1·82 | 1·88 | 2·19 | 1·74 | 1·32 | 2·16 | 1·45 | 1·51 | 1·54 |
| 0 | ? | 2·27 | 2·37 | 2·36 | 2·43 | 2·62 | 2·49 | 2·48 | 2·65 | 2·30 |
| 15 | 1·47 | 1·58 | 1·75 | 1·72 | 1·72 | 1·49 | 1·33 | 1·57 | 1·48 | 1·55 |
| 30 | 1·21 | 1·48 | 1·40 | 1·63 | 1·40 | 1·34 | 1·25 | ? | 1·35 | 1·38 |
| 45 | 1·20 | 2·02 | 1·47 | 1·58 | 1·38 | 1·42 | 1·24 | 1·46 | 1·47 | 1·31 |
| 60 | 1·30 | 2·38 | 1·42 | 1·40 | 1·66 | 2·0 | 1·59 | 1·55 | 2·60 | 1·54 |
| 75 | 1·53 | 2·74 | 1·48 | 3·26 | 4·42 | 6·4 | 1·95 | 2·0 | 2·84 | 1·95 |
| 90 | 1·55 | 2·81 | 1·44 | 3·92 | 6·40 | 6·53 | 2·06 | 3·2 | 3·16 | 3·7 |
| 105 | 1·40 | 3·19 | 1·57 | 4·16 | 5·73 | 6·14 | 2·08 | 5·42 | 2·48 | 2·89 |
| 120 | 1·47 | 2·3 | 1·75 | 2·34 | 3·48 | 6·25 | 1·50 | 4·79 | 2·23 | 2·30 |
| 135 | | 2·3 | | 1·95 | 2·68 | 3·84 | 2·79 | 5·20 | 2·50 | 2·15 |
| 150 | | 2·0 | | | 3·20 | 2·55 | 2·82 | 3·60 | 2·00 | 2·30 |

TOTAL ACID (ML. OF 0·1 N NaOH REQUIRED TO NEUTRALIZE 100 ML. OF STOMACH CONTENT)

| Time minutes | Control no drug | Atropine 1·3 mg. | Propionyl atropine methyl nitrate 2 mg. | 4 mg. | 6 mg. | 8 mg. | Valeryl atropine methyl bromide 5 mg. | 8 mg. | Atropine methyl bromide 8 mg. | Hyoscine methyl bromide 8 mg. |
|---|---|---|---|---|---|---|---|---|---|---|
| Fasting | 4·36 | 58·5 | 37·6 | 45·6 | 43·0 | 98·8 | 20·0 | 52·6 | 51·4 | 50·6 |
| 0 | ? | 9·6 | 10·0 | 10·0 | 52·0 | 5·0 | 3·5 | 7·8 | 5·2 | 10·6 |
| 15 | 50·4 | 58·0 | 36·0 | 49·6 | 40·6 | 44·0 | 60·5 | 37·0 | 42·4 | 38·6 |
| 30 | 50·0 | 26·2 | 68·4 | 67·4 | 64·8 | 70·8 | 79·0 | — | 63·0 | 58·2 |
| 45 | 54·2 | 25·6 | 78·6 | 76·0 | 49·0 | 67·6 | — | 52·0 | 51·6 | 70·6 |
| 60 | 55·4 | 24·0 | 78·0 | 52·4 | 3·2 | 40·0 | 44·2 | 44·0 | 37·0 | 49·6 |
| 75 | 68·0 | 29·0 | 75·4 | 11·2 | 8·2 | 5·0 | 10·2 | — | 16·0 | 31·0 |
| 90 | 61·8 | 36·0 | 49·6 | 11·4 | 3·2 | 5·0 | 25·8 | 7·6 | 15·6 | — |
| 105 | 48·2 | 41·8 | 58·0 | 9·2 | 6·4 | 8·4 | 14·8 | 8·8 | 23·0 | 16·6 |
| 120 | 60·6 | 40·2 | 42·0 | 16·8 | 11·6 | 5·6 | 56·6 | 11·6 | 26·4 | 24·0 |
| 135 | | | | 23·6 | 15·8 | 15·6 | 12·8 | 10·0 | | — |
| 150 | | | | | | 20·4 | 15·6 | 15·0 | | 70·0 |

## METHODS

*Acid secretion.* The antisecretory effect of a single oral dose on the secretion of the stomach in response to an alcohol test meal was estimated. The procedure was as follows.

All subjects fasted at least 8 hours previous to the test. Because of large individual variations in response to an alcohol test-meal each subject acted as his own control. The fasting content of the stomach was completely aspirated through a Rehfuss tube and any residual matter washed out with 20 ml. of distilled water at 37°. Immediately after this, the warmed test meal, with or without dissolved drug, was passed down the tube. A 10 ml. sample was then immediately withdrawn and thereafter a further 10 ml. every 15 minutes for 2 to 2½ hours. Estimations of free acidity, total acidity and pH were made on all samples including the

fasting sample. The acidities were obtained by titration with a 0·1N solution of NaOH using appropriate indicators; free acidity using Töpfer's reagent (0·5 per cent dimethylaminoazobenzene, end point approximately pH 2·8) and total acidity using phenolphthalein (end point approximately

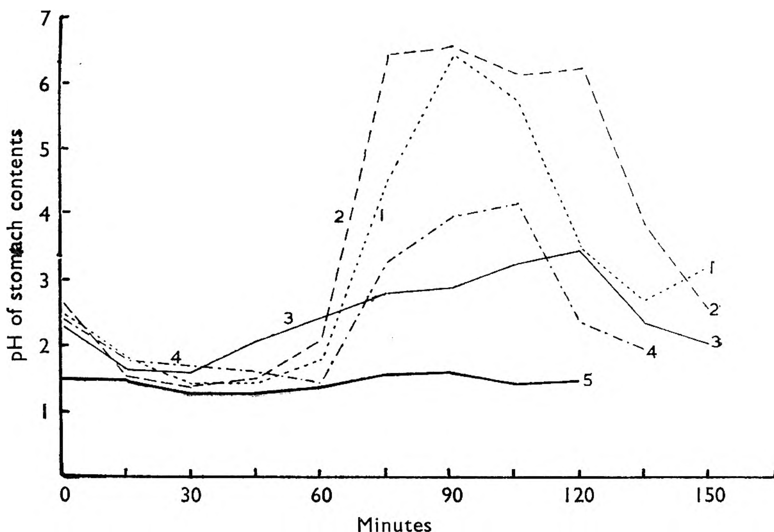


FIG. 1. Effect of various doses of PAMN of pH of human gastric contents. 1. PAMN 6 mg. 2. PAMN 8 mg. 3. Atropine 0·3 mg. 4. PAMN 4 mg. 5. Control.

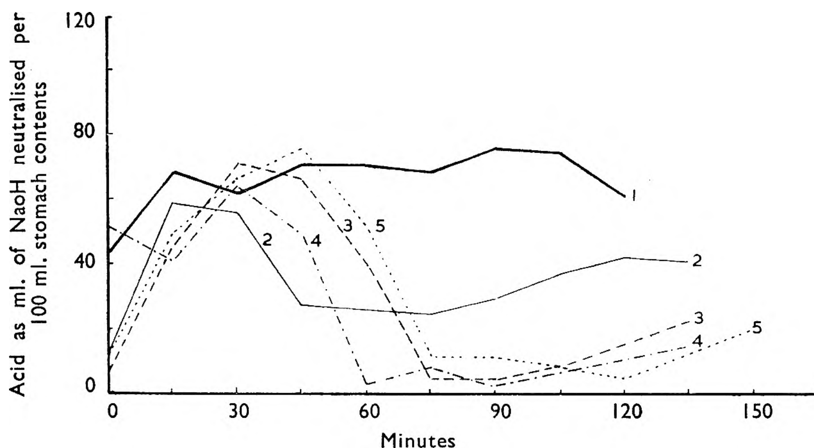


FIG. 2. Effect of various doses of PAMN on the acidity of the gastric contents (same subject as in Fig. 1). 1. Control 2. Atropine 0·3 mg. 3. PAMN 4 mg. 4. PAMN 6 mg. 5. PAMN 8 mg.

pH 7). All acid results were expressed as ml. of 0·1N NaOH required to neutralise 100 ml. of stomach content. As continuous aspiration apparatus was not available, the volume of secretion for each time interval could not be obtained.

*Motility.* Each subject acted as his own control, and different drugs were tested on separate days. The drugs were swallowed in tablet form with the aid of a little water 30 to 45 minutes before administration of the sugar. Immediately before the ingestion of 50 g. of the sugar (dissolved in 100 ml. of water), 0·05 ml. blood was taken from a

TABLE II

SUMMARY OF HUMAN GASTRIC SECRETION EXPERIMENTS

| Total number subjects tested | Drug single oral dose | Dry mouth (side effect) | Maximum pH (average) | Average duration of maximum pH minutes | Decrease of secretion (qualitative) |
|---|---|---|---|---|---|
| 6 | Atropine 0·65 mg. | ++ | 3·8 | 30 | + (?) |
| 5 | Atropine methyl bromide 8 mg. | ++ | 4·5 | 30 | ++ |
| 6 | Propionyl atropine methyl nitrate 8 mg. | + (?) | 4·9 | 60 | ++++ |
| 6 | Hyoscine methyl bromide 8 mg. | ++ | 3·2 | 30 | + (?) |

finger and 5 similar samples were taken at 15 minute intervals after the ingestion of the sugar. The glucose content of the blood samples was estimated by a sensitive colorimetric method requiring small samples of blood only. This facilitated the accurate timing of the blood samples. Each sample was diluted with distilled water (5 ml.) and the protein precipitated with $Zn(OH)_2$. The samples were then centrifuged and two

TABLE III

THE EFFECT OF DRUGS ON THE UPTAKE OF GLUCOSE AND SUCROSE FROM THE ALIMENTARY TRACT (MG. GLUCOSE/100 ML. BLOOD)

| Time minutes | 50 g. glucose | 50 g. glucose + 8 mg. PAMN | 50 g. glucose + 5 mg. pipenzolate | 50 g. glucose + 15 mg. propantheline | 50 g. glucose + 0·65 mg. atropine | 50 g. sucrose | 50 g. sucrose + 8 mg. PAMN |
|---|---|---|---|---|---|---|---|
| 0 | 103 | 101 | 82 | 95 | 109 | 90 | 95 |
| 15 | 102 | 119 | 109 | 108 | 132 | 87 | ? |
| 30 | 127 | 131 | 95 | 107 | 124 | 117 | 122 |
| 45 | 168 | 126 | 90 | 116 | 149 | 153 | 135 |
| 60 | 136 | ? | 103 | 105 | 114 | 139 | 120 |
| 75 | 136 | 102 | 84 | 100 | 104 | 115 | 130 |
| Maximum Rise | 65 | 30 | 27 | 21 | 40 | 63 | 27 |

aliquots (2 ml.) of clear supernatant liquid were taken. To each aliquot, chilled in an ice bath, a 3 ml. aliquot of anthrone solution (0·02 per cent in 95 per cent sulphuric acid) was added, and the colour of the mixture developed for exactly 7 minutes in a boiling water bath. Colour intensities were compared with the colour intensity of a known standard glucose solution using a Beckman spectrophotometer (wavelength 620 $m\mu$)[3].

## RESULTS

*Gastric acidity.* Comparisons of the test drug with atropine, atropine methyl bromide and controls were made in 76 experiments on 6 subjects. Tests with different drugs were done on different days. The variation noted from time to time in one individual was less than the variation between different subjects.

The results of a typical experiment are given in Table I and Figures 1 and 2. The results of 76 experiments on 6 persons in Table II. All subjects in whom 0·65 mg. atropine caused a rise in gastric pH and some decrease in acid secretion showed to the quaternary derivative a more pronounced response of longer duration. At pH 4·5 or more gastric pepsin is inactive. The blocking of the proteolytic enzyme greatly aids in the healing of gastric lesions. In most of the experiments in which the quaternary derivative was given, difficulty in obtaining some of the later 10 ml. samples was noted, indicating a qualitative decrease in secretion. This was not noted in either the control or atropine experiments.

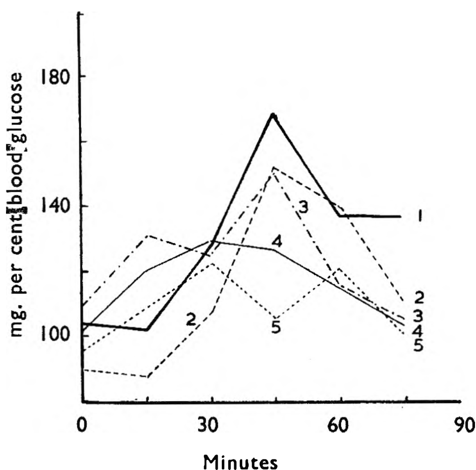


FIG. 3. Effect of PAMN on the uptake of glucose and sucrose from the human alimentary canal. 1. Glucose control. 2. Sucrose control. 3. Glucose × 0·65 mg. of atropine. 4. Glucose + 8 mg. of PAMN. 5. Glucose + 8 mg. of atropine.

*Motility.* 100 experiments on 8 persons were carried out. The results of a typical experiment are shown in Table III and Figure 3.

PAMN proved to be active in seven of the eight subjects tested. The results obtained from the eighth subject, a diabetic, were more variable but nevertheless in the majority of cases PAMN lessened the rise in blood sugar. The same tests were made on two of the subjects after the previous administration of 0·65 mg. atropine which proved to be about two thirds as effective as PAMN in each case, i.e. the rise in blood sugar after atropine was always less than that of the control but was greater than that obtained after PAMN. Thus PAMN produced a greater immobility of the alimentary tract and there was evidence that its action outlasted that of atropine.

*Effect on blood pressure and heart rate.* Ten volunteers were given 6 mg. of PAMN and the blood pressure and pulse rate measured for the next 3 hours. The results were variable, however the pulse rate increased by about five per minute in a number of instances. The systolic and diastolic pressures rose in some volunteers by about 5 mm. Hg. But the same variable results were obtained when the volunteers were tested again with placebos.

*Clinical Trial.* Two separate preliminary clinical trials have been made with PAMN at a dose of 6 mg. 6 hourly. No side actions were observed and no disturbances of micturition in any of the 26 patients. In both trials, one with 15, the other with 11 patients, the alleviation of symptoms has been satisfactory. Six patients have been treated for six months without any tolerance developing.

## Discussion

Two tests, applicable to human volunteers, were used to evaluate a quaternary ammonium derivative of atropine as a potential therapeutic agent for the relief of distress in peptic ulceration. The two tests were the alcohol test meal and the glucose tolerance test, the latter to determine the effect on alimentary motility. These gave results which are in remarkable agreement with one another, especially when made on the same subject.

The drug reduces the volume and acidity of the gastric juice and decreases the motility of the intestine. The decrease in volume of the gastric juice was often accompanied by an increase in mucous content as well as by a considerable rise in pH, which was too great to be attributed to swallowed saliva. Normal gastric secretion consists of a mixture of three secretions obtained from three distinct types of cells which may not be equally affected by the drugs. Perhaps in our case a greater inhibition of the acid-secreting cells than of the mucin-secreting cells was obtained. The secretion of the latter, which is alkaline in nature, also acts as a buffer because of its high protein content. The only untoward effect noticed has been a slight drying of the mouth in 19 experiments out of 171. There were no disturbances of vision or micturition. The slight degree of inhibition of salivation was noticed with doses of 8 mg. when the stomach was empty, a state which would increase the absorption of the drug. It was not noticed in patients receiving normal meals and at a dose of 6 mg.

The novel feature of the tests is the use of the glucose tolerance test to measure alimentary motility. The lower blood sugar levels which were obtained consistently when atropine or its derivatives were given, strongly supported our hypothesis that these reduced levels were due to lessened alimentary activity. Similar results obtained in the sucrose tolerance tests also supported this concept and as they were little, (if at all) different from the glucose tolerance tests it would appear that acid hydrolysis of the cane sugar in the stomach contributed little to the results.

The experiments on the diabetic volunteer were also interesting. This patient was an experienced research worker who fully understood the nature of his condition and was well stabilised. Fifteen experiments (alcohol test meals and glucose tolerance tests) were carried out, and in 6 cases the volunteer experienced an hypoglycaemic attack either during, or after the performance of the test. This would strongly suggest that the PAMN was delaying the uptake of sugar, presumably by decreased motility. Such evidence provides further support for our contention that the glucose tolerance test may be used as an index of alimentary motility.

Lieber[4] has compared thoroughly the effects of atropine, propantheline (Probanthine) and oxyphenonium (Antrenyl) on the post insulin and basal gastric secretion. At the same time he has studied the antisecretory activity and the side actions due to parasympathetic inhibition. Propantheline has most effect in accelerating the pulse. The other side actions parallel the effect on the heart and in decreasing order of activity of drug are propantheline, oxyphenonium and atropine. The gastric antisecretory activity does not follow the effects on salivary secretion etc.

For comparable antisecretory effect atropine has the least side actions. As our results show that, for equivalent antisecretory effect, PAMN has less side effects than atropine one would expect that PAMN has less side actions than either propantheline or oxyphenonium. Preliminary trials bear this out.

Finally we have not as yet tried the effect of PAMN on the "basal" secretion. In many ways "basal" secretion is a logical rather than physiological concept, in that it depends upon a definition. Lieber[4] has shown that atropine lessens the basal secretion and as PAMN is a close chemical derivative of atropine and has been shown to retain the anti-muscarinic actions of atropine[1] it might be assumed that PAMN would have a similar inhibitory action on the basal secretion.

PAMN would appear to have little effect on the circulatory system, both in volunteers and peptic ulcer patients.

*Acknowledgements.* We wish to thank Dr. Ian Wood, Director of the Clinical Research Unit, Royal Melbourne Hospital, for the preliminary notes of the first clinical trial and Dr. S. Gershon for his assistance in the second trial.

Propionyl atropine methyl nitrate is subject to Australian Patent No. 23270/56 and other patents.

## References

1. Herman, Shaw and Rosenblum, *J. Pharm. and Pharmacol.*, 1958, **10**, 348.
2. Bachrach, Rowen, Halsted, Schapiro, Holmström and Price, *Acta med. scand.*, Supp. 288, 1954.
3. Prokovnik and Nelson, *Aust. J. Exp. Biol.*, 1953, **31**, 279.
4. Lieber, *Acta Gastro-Enterol. Belgica*, 1956, **12**, 819.

# THE HYDROLYSIS OF ACETYLSALICYLIC ACID FROM AQUEOUS SUSPENSION*

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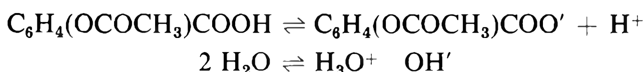
An investigation of the kinetics of the hydrolysis of acetylsalicylic acid from aqueous suspension is described. The net reaction is one of solution of the solid followed by hydrolysis in solution, so that the rate of reaction is dependent on the concentration of the saturated solution. Under conditions where the solubility of acetylsalicylic acid is small in comparison with the quantity in suspension, a zero order reaction results. The B.P.C. mixture is discussed.

The decomposition of acetylsalicylic acid solutions in water has been studied by several workers[1–6], who have shown that the compound is hydrolysed to form acetic and salicylic acids. The hydrolysis is a first order reaction, the net velocity being a function of the six possible reactions between the equilibria[7],



$$C_6H_4(OCOCH_3)COOH \rightleftharpoons C_6H_4(OCOCH_3)COO' + H^+$$
$$2\ H_2O \rightleftharpoons H_3O^+ \quad OH'$$

In consequence of this rapid hydrolysis from solution, the use of aqueous suspensions is preferred, although these too are known to decompose[8].

Examination of reactions between a suspended solid and its suspending medium requires a consideration of dissolution. The first measurements of dissolution were made by Noyes and Whitney[9], who observed the dissolution of benzoic acid and lead chloride respectively from a cylinder of the solid rotating in water. This was extended to chemical reaction by others[10,11], one of whom, Senter[12], said that the speed of the heterogeneous reaction depended on the rate of diffusion of the saturated solution from the solid, and the rate of the resulting homogenous chemical reaction. If either of these was appreciably slower than the other, its rate would determine that of the complete reaction. Under such conditions Senter[13] suggested that the effect of temperature on the reaction would indicate the relative rates, since the velocity of a chemical reaction is greatly influenced by temperature, an increase of 10° causing the rate of reaction to increase two or three fold, while the diffusion rises only 2·5 per cent per degree.

## EXPERIMENTAL

A procedure introduced by Tsokalatos and Horsch[2] was used whereby the degree of hydrolysis was calculated from the increase in acidity of the system. Titrations were carried out with sodium hydroxide in the presence of ethanol, using phenol red as indicator.

* The subject matter of this communication forms part of a thesis accepted by the University of Wales for the degree of Master of Pharmacy.

*Effect of Temperature*

Rates of hydrolysis of acetylsalicylic acid in mixture of acetylsalicylic acid B.P.C. were determined and compared at temperatures ranging from room temperature (approximately 20°) to 100°.

*Decomposition at room temperature and at* 34°. A quantity of mixture of acetylsalicylic acid B.P.C. stored in a glass stoppered bottle in a dark

TABLE I

HYDROLYSIS OF ACETYLSALICYLIC ACID SUSPENSIONS AT VARIOUS TEMPERATURES

| Room Temperature | | 34° | | 50° | | 60° | | 70° | | 100° | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Time in days | Per cent $C_9H_8O_4$ remaining | Time in days | Per cent $C_9H_8O_4$ remaining | Time in hours | Per cent $C_9H_8O_4$ remaining | Time in hours | Per cent $C_9H_8O_4$ remaining | Time in hours | Per cent $C_9H_8O_4$ remaining | Time in minutes | Per cent $C_9H_8O_4$ remaining |
| 4 | 98 | 7 | 93 | 1 | 99 | 1 | 93 | 1 | 91 | 15 | 85 |
| 7 | 96 | 15 | 87 | 2 | 98 | 2 | 90 | 2 | 81 | 30 | 71 |
| 13 | 95 | 21 | 78 | 3 | 96 | 3 | 89 | 3 | 73 | 45 | 62 |
| 19 | 93 | 29 | 76 | 4 | 94 | 4 | 85 | 4 | 65 | 60 | 52 |
| 26 | 93 | 36 | 66 | 5 | 92 | 5 | 82 | 5 | 59 | 75 | 42 |
| 47 | 89 | 43 | 60 | 6 | 92 | 6 | 75 | 6 | 54 | 90 | 36 |
| 62 | 89 | | | 7 | 91 | 7 | 71 | 7 | 47 | 120 | 19 |

cupboard (room temperature) and incubator (34°) was shaken daily, and sampled when required by removing approximately 5 g. in a tared weighing bottle.

*Decomposition at* 50°, 60°, *and* 70°. The reaction mixture was immersed in a constant temperature water bath and stirred continuously with a

TABLE II

RELATIVE VELOCITIES OF HYDROLYSIS OF MIXTURE OF ACETYLSALICYLIC ACID B.P.C. AT VARIOUS TEMPERATURES

| Temperatures °C. | Mean velocity quotient (per 10°C.) |
|---|---|
| 20 to 34 | 2·0 |
| 50 to 60 | 2·0 |
| 60 to 70 | 2·1 |
| 70 to 100 | 3·9 |
| 34 to 70 | 67 |

TABLE III

SOLUBILITY AND DISSOLUTION OF ACETYLSALICYLIC ACID

| Temperature °C. | Solubility (percentage w/w) | Relative dissolution rate |
|---|---|---|
| 20 | 0·33 | 1 |
| 34 | 0·50 | 1·5 |
| 50 | 1·0 | 3 |
| 60 | 1·5 | 4·5 |
| 70 | 3·3 | 10 |

mechanical stirrer. Approximately 5 ml. samples were withdrawn hourly into tared weighing bottles containing 15 ml. of cold water and weighed. The rapid cooling of the suspension thus, considerably retarded the rate of hydrolysis.

*Decomposition at* 100°. The same procedure was used as at 70°, the constant temperature being provided by a boiling water bath. Owing to the speed of the reaction, samples were withdrawn at 15 minute intervals, and as time did not permit duplicates, single determinations were made.

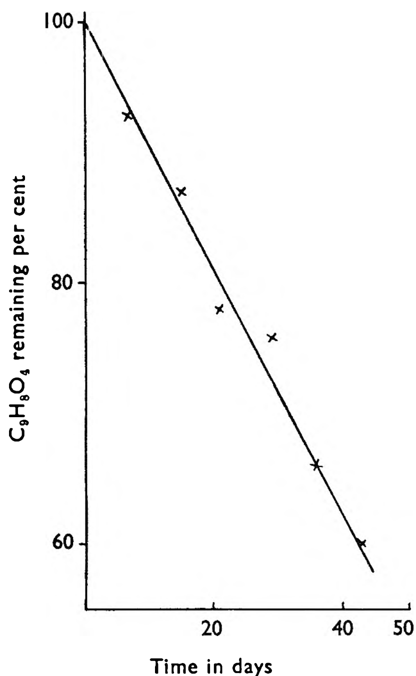



FIG. 1. Hydrolysis of mixture of acetylsalicylic acid at 34°.

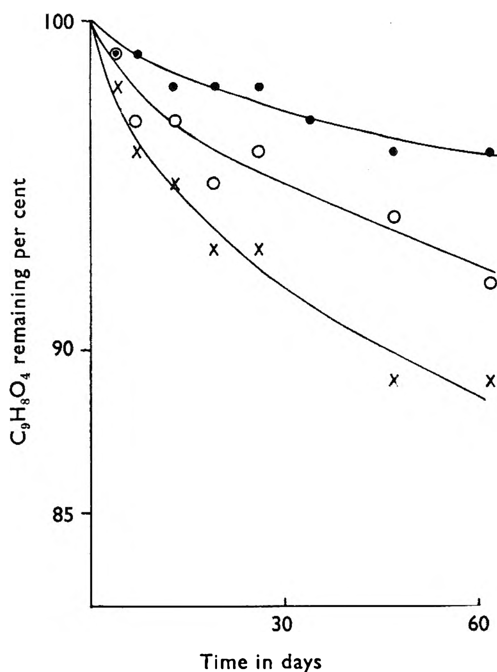



FIG. 2. Hydrolysis of acetylsalicylic acid suspensions at room temperature.

X Containing 3·3 per cent $C_9H_8O_4$ on preparation.
O " 6·5 " " " "
● " 13·0 " " " "

Results are summarised in Table I, and show the pronounced effect of temperature on the rate of reaction, 10 per cent hydrolysis occurring in only 15 minutes at 100°, and after 62 days at room temperature.

*Effect of Concentration*

Three suspensions were prepared, corresponding to mixture of acetylsalicylic acid B.P.C., and mixtures with double and quadruple the B.P.C. quantity of acetylsalicylic acid respectively. The preparations were stored in 500 ml. glass stoppered bottles in a dark cupboard, shaken daily, and the hydrolysis followed as before.

Three similar suspensions were also examined at 70°, and two suspensions, a single, and a double strength, at 100°.

*Solubility Determinations*

Solubilities were determined spectrophotometrically by a method described by Edwards[7].

## Discussion

In order to compare the rates of hydrolysis at the various temperatures, graphs were plotted relating the percentage of undecomposed acetylsalicylic acid to time of reaction, and from these the percentage hydrolysis at selected times determined. Velocity quotients were calculated from this information as the relative increase in hydrolysis per 10° temperature rise. These values are shown in Table II. The anticipated diffusion velocity quotient for 10° would be $(0{\cdot}025 \times 10) + 1 = 1{\cdot}25$, which is less than the smallest of the experimental values. Further, although the two to threefold increase for chemical reactions quoted by Senter is of the same order as most of the velocity quotients, it bears no comparison with the velocity quotient obtained between 34° and 70°. A clue to this very large figure was found in the observation that the suspensions became translucent at about 70°, indicating that the solubility of acetylsalicylic acid had increased with temperature, until at 70° all the acid in the mixture containing 3·3 per cent had dissolved. This is confirmed by the solubility figures, shown in Table III.

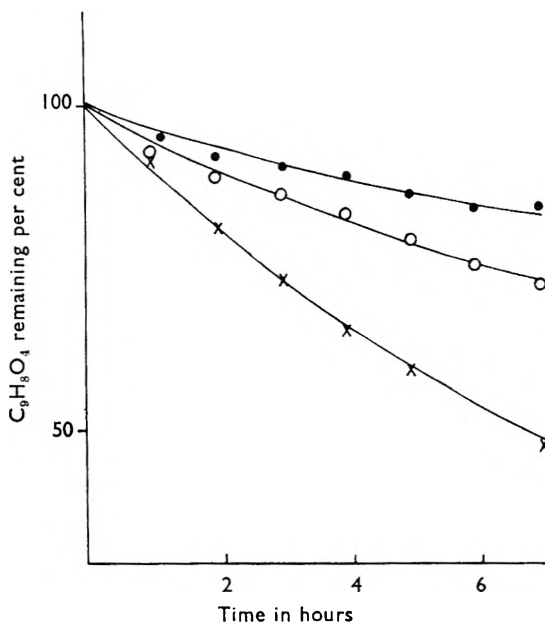



Fig. 3. Hydrolysis of acetylsalicylic acid suspensions at 70°.

X Containing 3·4 per cent $C_9H_8O_4$ on preparation.
O ,, 6·9 ,, ,, ,, ,,
● ,, 12·9 ,, ,, ,, ,,

The Noyes Whitney equation:—

$$\frac{dc}{dt} = k(c_S - c)$$

where c is the concentration of the continuous phase, and $C_S$ that of the saturated solution, predicts that the rate of dissolution increases with solubility. Relative rates of dissolution, calculated when t and c are zero, are given in Table III, and indicate that increase in dissolution alone cannot be responsible for the high velocity quotients.

Figure 1 shows the curve of concentration against time for the hydrolysis at 34°. It is a straight line, which indicates that the rate of hydrolysis is independent of the quantity of acetylsalicylic acid in suspension, or in

other words, is of zero order. This suggests that the reaction at 34° is in two parts, (*a*) solution of acetylsalicylic acid, followed by, (*b*) decomposition of the acetylsalicylic acid in solution. As the acid in solution decomposes, more dissolves, so that the rate of hydrolysis depends on the strength of the saturated solution. The hydrolysis of the suspension is therefore slow because the acetylsalicylic acid in solution only, is subject to hydrolysis. Since the solubility is constant the rate of hydrolysis is independent of the total quantity of acetylsalicylic acid, and the reaction is zero order. In the light of this mechanism, the marked increase of reaction rate with temperature is not surprising.

If the reaction is zero order, the more concentrated a suspension the smaller would be the proportion of acetylsalicylic acid hydrolysed, so that the stability of such suspensions should increase with concentration. By comparing the rates of hydrolysis of suspensions of different strengths, the zero order reaction can be verified, and more stable preparations of acetylsalicylic acid formulated.

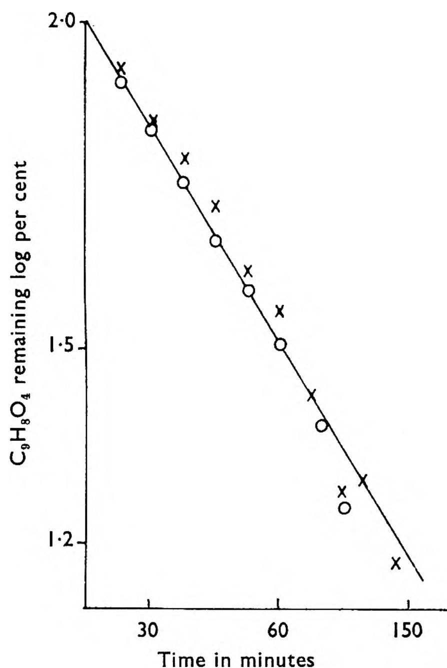



FIG. 4. Hydrolysis of acetylsalicylic acid suspensions at 100°.
X Containing 3·3 per cent $C_9H_8O_4$ on preparation.
O ,, 6·9 ,, ,, ,,

The rates of hydrolysis of suspensions containing 3·3, 6·5, and 13·0 per cent of acetylsalicylic acid at room temperature are shown in Figure 2. There was a definite increase in stability with concentration, at every stage of the determination the degree of hydrolysis was in the same order as the concentrations, and after 62 days storage the percentages of unchanged acetylsalicylic acid were 90, 94 and 97 per cent respectively, relative rates of hydrolysis being 10:6:3.

The comparative rates of hydrolysis of similar suspensions at 70°, shown in Figure 3, gave the same picture, but at 100° the rates of hydrolysis of the two suspensions were identical, and the hydrolysis curves superimposable (Fig. 4).

In the hydrolysis at room temperature and at 70° the percentage hydrolysis in a given time depends on, and is approximately proportional to the concentration of the suspension. The hydrolysis curves for the determinations at room temperature do not follow the straight line of the

zero order reaction, but the plot of log concentration against time showed that the reaction is also not first order at any concentration.

At 70° the hydrolysis curves show less curvature than at room temperature, and approach the straight line of the zero order reaction as the concentration is increased. In the log concentration against time curves in Figure 5, the 3·4 per cent suspension follows a straight line, indicating

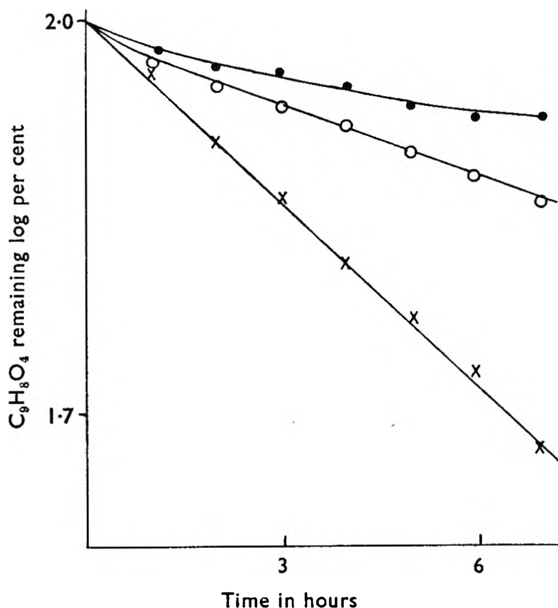



FIG. 5. Hydrolysis of acetylsalicylic acid suspensions at 70°.

X containing 3·4 per cent $C_9H_8O_4$ on preparation.
O ,, 6·9 ,, ,, ,, ,,
● ,, 12·9 ,, ,, ,, ,,

that the reaction is first order. Most of the reaction with the 6·9 per cent suspension behaves in the same way, while the 12·9 per cent suspension exhibits a definite curve. The curves in the figure suggest the presence of a first order reaction, but with the more concentrated suspension this is less marked. The inference however confirms that the solubility of acetylsalicylic acid at 70° lies below 6·9 per cent, that the 3·4 per cent suspension decomposes unimolecularly, and that as the quantity of acetylsalicylic acid in suspension increases above 3·4 per cent, the effect of the first order reaction becomes less in relation to the zero order reaction of solution followed by hydrolysis.

The behaviour of the reaction at 100° was typically first order, so that concentration had no effect on the progress of the reaction.

The aim of this work has been to determine in what way acetylsalicylic acid hydrolyses from aqueous suspension, why the suspension is more stable than the solution, and what steps can be taken to further stabilise the suspension.

It was intended that the work be comparative and not quantitative. To give absolute figures for hydrolysis rates, particle size and degree of agitation would have to be defined. These conditions were standardised in as much as the same sample of acetylsalicylic acid was used throughout, together with the same stirrer running at the same speed, but neither property was measured.

The rate of hydrolysis has been studied with several samples of mixture of acetylsalicylic acid B.P.C., mainly as control in storage tests on other suspensions, and the degree of hydrolysis has varied from 1 to 4 per cent after 7 days at room temperature. Similarly, 3 to 5 per cent and 5 to 6 per cent were found to have hydrolysed after 14 and 21 days respectively. The limit test of the British Pharmacopoeia allows a maximum of 0·05 per cent of salicylic acid in acetylsalicylic acid, and 0·15 per cent in the tablets, so that the mixture would cease to comply with these requirements after less than one day's storage.

Temperature has been shown to have marked effect on the rate of hydrolysis, a rise from 20° to 34° resulting in a one-and-a-half to twofold increase. It is therefore important that the mixture be kept in a cool place.

As the rate of hydrolysis is inversely proportional to concentration, a suspension containing four times the quantity of acetylsalicylic acid as the official mixture will hydrolyse only a quarter as much in a given time. By increasing the concentration of the acid in the B.P.C. mixture fourfold the stability can be increased to the same extent and the mixture would lose less than 1 per cent of its potency in one week.

*Acknowledgements.* The author thanks the Welsh School of Pharmacy for the facilities given him, and Mr. P. H. Griffiths, B.Sc., F.R.I.C., for his assistance throughout the work.

## References

1. Rath, *Annalen*, 1907, **358**, 98.
2. Tsokalatos and Horsch, *Bull. Soc. Chim.*, 1914, **15**, 743; 1915, **17**, 401.
3. Smith, *Pharm. J.*, 1915, **94**, 200.
4. Wilson, *ibid.*, 1929, **122**, 196.
5. Dott, *ibid.*, 1930, **124**, 214.
6. Morton, *Quart. J. Pharm.*, 1933, **6**, 493.
7. Edwards, *Trans. Faraday Soc.*, 1950, **46**, 723.
8. Tomski and Waller, *Pharm. J.*, 1940, **144**, 53.
9. Noyes and Whitney, *Z. Physikal Chem.*, 1897, **23**, 689.
10. Nernst, *ibid.*, 1904, **47**, 52.
11. Sand, *Proc. Roy. Soc.*, 1905, **74**, 356.
12. Senter, *ibid.*, 566.
13. Senter, *J. Physikal Chem.*, 1906, **9**, 311.

# THE DETERMINATION OF SMALL QUANTITIES OF CHLORHEXIDINE IN PHARMACEUTICAL PREPARATIONS

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A colorimetric method has been developed for the determination of small quantities of chlorhexidine by reaction in alkaline solution with sodium hypobromite. The precipitation of the base is prevented by the use of a surface-active agent. The method is rapid and accurate and has been applied to a variety of formulated products containing chlorhexidine.

The antibacterial drug chlorhexidine (1 : 6-di($N'$-*p*-chlorophenylbiguanidide $N^5$)hexane) may be incorporated in various formulations in a range of concentrations. When present in relatively large amounts it may usually be estimated by extraction of the base followed by titration in a non-aqueous medium. Alternatively a spectrophotometric method may be applied to a suitably purified extract. If the concentration of chlorhexidine is 1 per cent or less, however, large amounts are required for the base extraction method and it is often difficult to prepare extracts which are sufficiently free from irrelevant absorption for a spectrophotometric determination to be possible. During a search for a more suitable procedure it was noted that hypochlorous acid reacts with chlorhexidine to give a transient red colour and it was thought that this might form the basis of a colorimetric method of assay.

## Experimental

A solution of chlorhexidine diacetate in dilute hydrochloric acid was prepared and diluted with water to contain 0·3 mg. of chlorhexidine per millilitre. Aliquots each of 5 ml. were treated separately with increasing amounts of a solution of sodium hypochlorite containing 1 per cent available chlorine and the effect assessed visually. Small volumes of hypochlorite gave an immediate blood-red colour which faded rapidly to pale yellow; with increasing volumes the blood-red colour persisted but decreased progressively in intensity. This effect was found to be due to the raising of the pH, caused by the alkali present in the sodium hypochlorite solution which precipitated chlorhexidine base before the reaction was complete. The colour produced in neutral or acid solution was too transient to be of practical value and an attempt was made to prevent the precipitation of chlorhexidine base in alkaline solution by the addition of a surface-active agent. With this object 5 ml. of a 20 per cent sodium lauryl sulphate solution was added to a 5 ml. aliquot of the chlorhexidine acetate solution, together with sufficient 0·1N sodium hydroxide solution to produce an excess alkalinity of 0·5 ml. On addition of 5 ml. of sodium hypochlorite solution an immediate blood-red colour was produced which faded very slowly. A slight turbidity was present, however, which

made the solution unsuitable for optical measurement and, since filtration led to adsorption of some of the colour to the filter paper, a search was made for a more suitable surface-active agent. Various compounds of anionic, cationic and non-ionic types were tried, involving about twenty compounds in all. Most of these were effective to a greater or lesser extent but cetrimide and phenactide (*β p-tert.*-octylphenoxyethyldiethylbenzylammonium chloride) were superior to the others, both in clarity of the resultant solution and the stability of the colour. On account of its ready availability cetrimide was chosen and was used in all subsequent work. Sodium hypochlorite solution is inconvenient to prepare and at this stage the more readily prepared hypobromite was tried as a substitute. Initial results were promising and it was decided to investigate the potentialities of the method using both reagents.

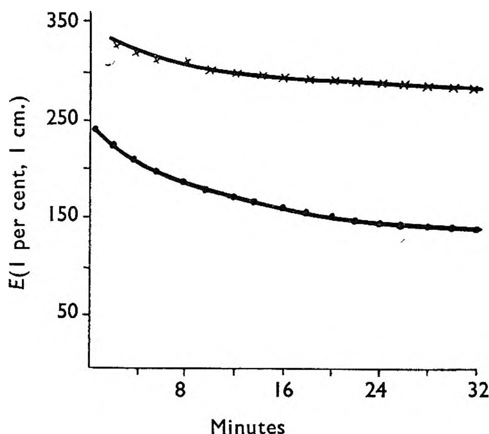


FIG. 1. Stability of colour with time.

X Alkaline sodium hypobromite 1 per cent w/v available bromine.

● Alkaline sodium hypochlorite 1 per cent w/v available chlorine.

*Selection of Optimum Conditions of Reaction*

*Stability of colour on standing at* 20°. Aliquots of 5 ml. of chlorhexidine acetate solution were diluted to about 80 ml. in each of two 100 ml. flasks and 5 ml. of 20 per cent cetrimide solution added. After the addition of 0·5 ml. 0·1N sodium hydroxide the colour was developed by adding 5·0 ml. of sodium hypochlorite solution to one, and 5·0 ml. of sodium hypobromite solution to the other, both solutions having 1 per cent of available halogen. The volume of each was adjusted to 100 ml. and the optical density measured at 480 m$\mu$ in 1 cm. cells at intervals over the succeeding 30 minutes. Results are shown in Figure 1 and illustrate the superiority of sodium hypobromite solution. In view of the enhanced stability of the colour formed with the latter reagent further work with sodium hypochlorite was abandoned. A standing time of 25 minutes was chosen as sufficient for the colour to reach stability.

*Variation of sodium hypobromite concentration.* The development of the colour was carried out as described in the preceding section varying the quantity of sodium hypobromite solution, the optical density of each solution being measured 25 minutes after the addition of the reagent. Results are given in Table I and show that variation of the sodium hypobromite content has no effect on the resultant colour.

*Variation of alkalinity.* The alkalinity of the sodium hypobromite solution was varied between 0·7 and 1·5N, other conditions remaining unchanged, without affecting the optical density of the colour produced. It was convenient to prepare the reagent in 1N sodium hydroxide and this concentration of alkali was used in all subsequent experiments.

*Variation of the cetrimide concentration.* The reaction was carried out using varying concentrations of cetrimide and the results are shown in

TABLE I

EFFECT OF VARIATION OF THE SODIUM HYPOBROMITE CONCENTRATION ON THE INTENSITY OF THE COLOUR

| ml. of sodium hypobromite 1 per cent available bromine | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| *E* (1 per cent, 1 cm.) 480 mμ .. | 302 | 303 | 303 | 303 | 303 | 304 |

Table II. It will be seen that for constant results to be obtained a minimum of 5 ml. of a 20 per cent solution must be used.

*Variation of temperature.* The temperature at which the solutions were allowed to stand during the 25 minutes development time was varied between 5° and 25°, and the results are shown in Table III. It is seen that temperature control is most important if reproducible results are to be obtained. As 20° is a readily attainable laboratory temperature subsequent determinations were performed at this temperature.

TABLE II

EFFECT OF VARIATION OF THE CETRIMIDE CONCENTRATION ON THE INTENSITY OF THE COLOUR

| ml. 20 per cent cetrimide solution | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| *E* (1 per cent, 1 cm.) 480 mμ .. | 294 | 294 | 298 | 303 | 308 | 310 | 310 | 311 |

## GENERAL METHOD

On the basis of the foregoing experimental work the following procedure was established for the determination of chlorhexidine in simple solution.

### *Reagents*

*Alkaline sodium hypobromite.* Dissolve 10 g. of sodium hydroxide A.R. in 400 ml. of distilled water in a 500 ml. flask and add, in small portions, 5·5 ml. of bromine A.R. stirring between each addition until the bromine has dissolved. Adjust the volume of the solution to 500 ml. with distilled water. Standardise the solution as follows. Pipette 10·0 ml. into a 250 ml. conical flask and add, in order, 25 ml. of distilled water, 2 g. of potassium iodide (free from iodate) and 10 ml. of glacial acetic acid. Titrate the liberated iodine with 0·1N sodium thiosulphate solution using starch as an indicator.

Based on the determined strength dilute the solution with distilled water to contain 1·5 per cent of available bromine. Prepare the reagent by mixing 66 ml. of the latter solution with 33 ml. of 3N sodium hydroxide solution. It is stable for about one month.

*Cetrimide solution.* Dissolve 20 g. of cetrimide B.P. in 80 ml. of warm distilled water, cool, and dilute to 100 ml.

*Method*

Transfer an aliquot of the solution, such as would be expected to contain about 1·5 mg. of chlorhexidine, to a 100 ml. volumetric flask and adjust the volume to about 80 ml. by the addition of distilled water. Add 5·0 ml. of cetrimide solution and, if the solution is acid, sufficient N sodium hydroxide solution to render alkaline to litmus paper, plus 0·5 ml. in excess. Mix well, add 1·0 ml. of *iso*propanol to suppress the froth and place the flask in a water bath at 20° ± 2°. When the temperature has reached equilibrium add 2·0 ml. of alkaline sodium hypobromite solution, adjust the volume to 100 ml., mix and replace in the water bath. At the

TABLE III

EFFECT OF TEMPERATURE VARIATION ON THE INTENSITY OF THE COLOUR

| Temperature °C | 5 | 10 | 15 | 20 | 25 |
|---|---|---|---|---|---|
| *E* (1 per cent, 1 cm.) 480 mμ .. | 348 | 338 | 317 | 307 | 294 |

same time perform a blank on the reagents used exactly as described above omitting only the sample. Allow the flasks to remain in the water bath for exactly 25 minutes and then immediately measure the optical density of both sample and reagent blank against distilled water in 1 cm. cells at 480 mμ on a suitable spectrophotometer. Correct the optical density of the sample by subtracting the reagent blank reading. Obtain the chlorhexidine content of the sample by reference to a calibration graph prepared by repeating the above procedure on solutions containing known amounts of chlorhexidine acetate. A suitable graph is obtained using 2, 4, 6, 8 and 10 ml. aliquots of a 0·0003 g./ml. solution of chlorhexidine acetate.

*Application of the Method to Formulated Products*

*Compound solutions of chlorhexidine.* In all samples examined it was possible to use the foregoing method without modification, the effect of added colouring matter being negligible at the dilution used and the usual perfuming agents being without effect on the development of the colour.

*Creams and ointments.* It is necessary to extract chlorhexidine from the fatty excipients of the sample before the development of the colour with sodium hypobromite. The following technique was found generally applicable. Transfer an accurately weighed quantity of the sample, expected to contain about 30 mg. of chlorhexidine, to a 150 ml. separating funnel with the aid of 20 ml. of distilled water and add 10 ml. of 1N hydrochloric acid. Extract successively with three portions, each of 25 ml. of chloroform, combining the chloroform extracts in a second separating funnel. Wash the combined chloroform extracts with two 10 ml. portions of water, adding these washings to the acid liquid in the first separating funnel. Discard the chloroform extracts. Transfer the combined acid layer and washings to a 100 ml. volumetric flask and adjust

to the mark with distilled water. Determine the chlorhexidine content on a 5 ml. aliquot of the above solution by the method described for simple solutions.

*Lozenges.* Transfer an accurately weighed quantity of powdered lozenges, expected to contain about 5 mg. of chlorhexidine to a 150 ml. separating funnel with the aid of 20 ml. of distilled water. Add 10·0 ml. of 3N sodium hydroxide and extract with three successive portions, each of 25 ml., of ether, combining the ether extracts in a second separating funnel. Extract the combined ether extracts with three successive 25 ml. portions of 0·1N hydrochloric acid, filtering each in turn through a No. 1 Whatman filter paper into a 100 ml. volumetric flask. Wash the filter paper with 20 ml. of distilled water, adding the washings to the acid layers in the 100 ml. flask. Adjust to volume with distilled water and mix. Determine the chlorhexidine on a 25 ml. aliquot of the latter solution exactly as described for simple solutions of chlorhexidine.

TABLE IV

SUMMARY OF RESULTS ON SAMPLES EXAMINED

| Sample | No. | Chlorhexidine content | | | | |
|---|---|---|---|---|---|---|
| | | Nominal | Proposed method | | Alternative method | |
| | | per cent w/v | per cent w/v | | * per cent w/v | |
| Liquid antiseptic .. .. | 1 | 0·30 | 0·30 | 0·29 | 0·29 | 0·29 |
| | 2 | 0·29 | 0·29 | 0·28 | 0·27 | 0·27 |
| | 3 | 0·30 | 0·31 | 0·30 | 0·30 | 0·31 |
| | 4 | 0·30 | 0·29 | 0·29 | | |
| | 5 | 0·30 | 0·29 | 0·29 | | |
| | 6 | 0·30 | 0·30 | 0·30 | | |
| | 7 | 0·30 | 0·31 | 0·31 | | |
| | 8 | 0·30 | 0·29 | 0·30 | | |
| | | per cent w/w | per cent w/w | | † per cent w/w | |
| Antiseptic cream .. .. | 9 | 1·03 | 1·05 | 1·06 | 1·04 | 1·02 |
| | 10 | 1·00 | 0·98 | 0·96 | 0·96 | 0·96 |
| | 11 | 1·00 | 0·98 | 1·00 | 0·98 | 0·98 |
| | 12 | 1·00 | 0·97 | 0·99 | 0·98 | 1·00 |
| Obstetric cream .. .. | 13 | 1·00 | 1·04 | 1·05 | 1·04 | 1·04 |
| | 14 | 1·00 | 0·99 | 0·97 | 0·93 | 0·94 |
| | 15 | 1·00 | 1·03 | | | |
| | 16 | 1·00 | 0·98 | | | |
| | 17 | 1·00 | 1·00 | | | |
| | 18 | 1·00 | 1·02 | | | |
| | | mg./lozenge | mg./lozenge | | | |
| Antiseptic lozenges .. .. | 19 | 5·0 | 4·95 | 4·95 | | |
| | 20 | 5·0 | 4·95 | 5·04 | | |
| | 21 | 5·0 | 4·85 | 5·00 | | |
| | 22 | 5·0 | 4·9 | 4·9 | | |
| | 23 | 5·0 | 5·0 | 4·9 | | |
| | 24 | 5·0 | 4·85 | 4·9 | | |

Samples 1, 2, 9, 10, 13 and 14 were prepared in the laboratory from materials of established purity.
* A spectrophotometric procedure was used, a reagent blank being performed on all materials used in the formulation.
† The procedure used was titration of the extracted base with perchloric acid in glacial acetic acid.

The procedure described above is suitable for lozenges containing the usual excipients and flavouring agents. The presence of a local anaesthetic of the *p*-aminobenzoic ester type, for example benzocaine, does not cause interference, but if a quaternary ammonium bactericide is present the chlorhexidine is not completely extracted and a modified procedure is necessary. Results on this and previously described formulations are given in Table IV.

*Acknowledgements.* The author wishes to express his thanks to Mrs. A. Swift and Mr. H. E. Mill for assistance with the experimental work.

# A COMPARATIVE STUDY ON SOME PHARMACOLOGICAL EFFECTS OF DIGITOXIN, ACETYLDIGITOXIN AND GITALIN (AMORPHOUS) IN CATS

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Received February 17, 1958

The lethal doses of digitoxin, acetyldigitoxin and gitalin were determined in cats. Electrocardiograms were recorded intermittently during the course of the repeated injections of these glycosides. Their relative lethal activities in cats were found to be similar to the reported relative therapeutic activities in man. Gitalin was found to be significantly more liable to cause cardiac arrhythmias than the other two glycosides.

It is generally known that the therapeutic indices of various cardiac glycosides are essentially identical although the absolute activities are different[1–4]. More recently, however, a number of investigators[5,6] have reached the conclusion from clinical experience that gitalin (amorphous) has a greater therapeutic index than the other cardiac glycosides. Whether this reported difference in the therapeutic index can be demonstrated in experimental animals, was herein examined.

## Methods

The lethal doses of digitoxin, acetyldigitoxin and gitalin were determined in adult healthy cats weighing 1·8 to 3·5 kg. The procedure was similar to the assay for digitoxin described in the U.S. Pharmacopoeia XIII. In brief, the glycoside, in solution, was given intravenously at a rate of 1 ml./kg. every 5 minutes. The concentration of the solution was such that the cat would die after receiving 13 to 19 injections of the glycoside. A total of 14 cats were used for each glycoside. During the course of the experiments, electrocardiograms (Lead II) of these cats were recorded before each injection. The digitoxin was a sample of the U.S.P. Reference Standard, and the acetyldigitoxin and gitalin were supplied by Sandoz Ltd., and White Laboratories, respectively.

## Results

The mean lethal dose in mg./kg. and its standard error were calculated for each glycoside from the concentration of the solution and the number of injections used. The results are listed in Table I. The figures in parentheses are quoted from Rothlin and others[7]. The relative lethal activities were computed from the mean lethal doses, the activities of acetyldigitoxin and gitalin being expressed in percentages in terms of digitoxin. While the lethal doses of digitoxin and acetyldigitoxin determined by Rothlin and others appear to be appreciably smaller than our figures, the relative activities of these two glycosides ascertained from their data are in good agreement with ours.

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For comparative purposes, the relative therapeutic activities of these glycosides are also listed in Table I. According to the data of Willems[8], acetyldigitoxin has 80 or 93 per cent of the activity of digitoxin depending on the rate of digitalization, and from Batterman and others[5] gitalin has 42·8 per cent of the activity of digitoxin. The relative lethal activities of

TABLE I

THE RELATIVE ACTIVITY OF SOME CARDIAC GLYCOSIDES

| Glycoside | Lethal dose* | Relative lethal activity in cats per cent | Relative therapeutic activity in man per cent |
|---|---|---|---|
| Digitoxin | 0·463 ± 0·014 (0·386) | 100·0 | 100·0 |
| Acetyl-digitoxin | 0·556 ± 0·015 (0·447) | 82·2 (86·4) | 80·0† 93·0† |
| Gitalin | 1·154 ± 0·033 | 39·6 | 42·8‡ |

Figures in parentheses are quoted from Rothlin and others[7].

* Mean ± S.E., in mg./kg.

† From data reported by Willems[8].

‡ From data reported by Batterman and others[5].

these three glycosides in cats are similar to their relative therapeutic activities in man.

As expected, a variety of changes was observed in the ECG. Since the control heart rates of the cats differed considerably, in order to increase the precision of the result, the chronotropic effects of these glycosides were expressed as changes in heart rate. The means and standard errors of the changes in heart rate after the cats had received approximately

TABLE II

THE CHRONOTROPIC EFFECTS OF SOME CARDIAC GLYCOSIDES IN CATS

| Glycoside | After 25 per cent of LD | After 40 per cent of LD | After 55 per cent of LD | After 70 per cent of LD | After 85 per cent of LD |
|---|---|---|---|---|---|
| Digitoxin .. | 97·2 ± 1·5 | 100·5 ± 3·1 | 97·1 ± 3·3 | 115·8 ± 5·5 | 133·1 ± 8·5 |
| Acetyldigitoxin | 97·4 ± 1·5 | 95·7 ± 2·6 | 100·4 ± 6·8 | 101·3 ± 3·6 | 118·4 ± 5·5 |
| Gitalin .. | 92·5 ± 2·2 | 96·0 ± 4·6 | 101·0 ± 6·3 | 113·9 ± 6·3 | 121·7 ± 6·2 |

The figures are mean ± S.E. of heart rates expressed as percentages of the control value in each cat.

25, 40, 55, 70 and 85 per cent of the lethal doses of these glycosides are listed in Table II. It may be seen that the negative chronotropic effects of these glycosides, on the average, were not marked. But definite bradycardia (a reduction in heart rate of 15 per cent or more) did occur in about one third of the cats in each group and prolongation of the PR interval appeared only in these animals. Furthermore, in all the cats that had bradycardia, the minimum heart rate was recorded after they had received approximately 60 per cent of the lethal dose of the glycoside. Figure 1 shows the ECG of the cat that had the most marked bradycardia. This cat died after receiving 17 injections of gitalin. Immediately before the eleventh injection the heart beat was reduced from a control of 148 to 70 per minute, and simultaneously the PR interval increased from 0·10 sec.

to 0·16 sec. A delayed increase in heart rate was seen in every cat, as shown in Table II.

In addition to the chronotropic effects of the glycosides, many other changes were noted in the ECG. The most consistent were cardiac arrhythmias. Extra systoles were usually seen at low dosages. At

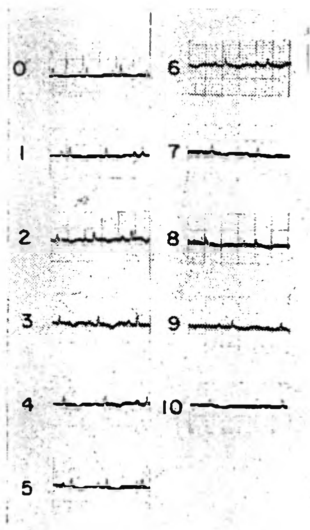


FIG. 1. The ECG of a cat showing marked bradycardia and prolongation of the PR interval. The cat died after receiving 17 injections of gitalin The control heart rate was 148 per. minute and the PR interval, 0·10 sec. Five minutes after the tenth injection, these changed to 70 per minute and 0·16 second respectively.

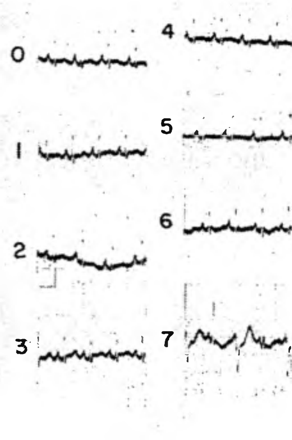


FIG. 2. The ECG of a cat which died after receiving 15 injections of digitoxin. There was no definite bradycardia or prolongation of the PR interval. Extrasystoles appeared after the seventh injection.

higher dosages, there were pulsus bigeminus, pulsus trigeminus, and various types of tachycardia. The dose that induced the first sign of arrhythmia, expressed as a percentage of the lethal dose for each cat, was determined. For each glycoside the mean and standard error of this dose

TABLE III

DOSES OF GLYCOSIDES INDUCING CARDIAC ARRHYTHMIAS AND CHANGES IN T WAVE, EXPRESSED AS THE LETHAL DOSES PER CENT IN CATS. (MEAN ± S.E.)

| Glycoside | Cardiac arrhythmia (lethal dose, per cent) | Changes in T wave (lethal dose, per cent) |
|---|---|---|
| Digitoxin .. | 67·9 ± 5·2 | 38·3 ± 3·1 |
| Acetyldigitoxin | 70·9 ± 2·5 | 46·1 ± 4·1 |
| Gitalin .. | 44·3 ± 4·0 | 34·4 ± 2·4 |

in all 14 cats are listed in Table III. With digitoxin and acetyldigitoxin, cardiac arrhythmias occurred only after two thirds of the lethal dose was given, whereas with gitalin these occured before one half of the lethal dose

was given. Heart block was usually seen shortly after the onset of arrhythmias. Bundle branch block in most cases appeared after A–V block. The T wave became bisphasic or inverted in many cats. The doses that caused changes in the T wave, expressed as percentages of the lethal doses of these glycosides, are also summarized in Table III.

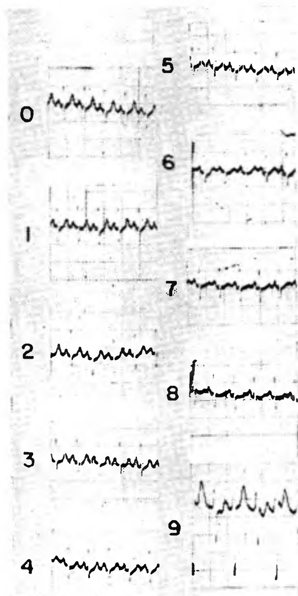


FIG. 3. The ECG of a cat which died after receiving 13 injections of acetyldigitoxin. There was no definite bradycardia or prolongation of the PR interval. Extrasystoles appeared after the ninth injection.

Other ECG changes such as lowering of the P wave and the QRS complex and shortening of the QT interval were inconsistent. Depression of the ST segment occurred in 7 of the cats which had received, on the average, 52·1 per cent of the lethal dose of gitalin. Elevation, instead of depression, of the ST segment was observed in 9 of the cats receiving acetyldigitoxin and in only 3 of the cats receiving digitoxin. With acetyldigitoxin, ST elevation occurred after 49·0 per cent of the lethal dose was given. This is not significantly different from gitalin. Figures 2 and 3 illustrate the absence of a negative chronotropic effect. Figure 4 exemplifies the earlier appearance of extra systoles in cats receiving gitalin in contrast to those receiving digitoxin and acetyldigitoxin (cf. Fig. 2 and 3).

The therapeutic ratio for these glycosides, as reported in the literature, are given in Table IV. These figures show that gitalin has a wider margin of safety than the other two glycosides. In cats, if it may be assumed that the therapeutic dose corresponds to one third of the lethal dose as suggested by Walton[9], the ratio of this "therapeutic dose" to the arrhythmia dose presents an exactly opposite picture. In other words, gitalin is more liable to cause cardiac arrhythmias than the other two glycosides.

TABLE IV

THE "THERAPEUTIC RATIOS" OF SOME CARDIAC GLYCOSIDES

| Glycoside | "Therapeutic ratio" in cats per cent | "Therapeutic ratio" in man per cent |
|---|---|---|
| Digitoxin .. | 49·1 | 58·0* |
| Acetyldigitoxin | 47·0 | 53·0† |
| Gitalin .. | 76·9 | 36·9* 42‡ |

* Figures from Batterman and others[6].
† Figure from Loeffler and others[12].
‡ Figure from Weiss and Steigmann[6].

## Discussion

While the lethal doses and the cardiac arrhythmia doses of these glycosides in cats were determined with satisfactory precision, a suitable criterion for therapeutic doses was not observed. The lack of a consistent bradycardiac effect of these glycosides in our experiments agrees with the findings of Gold and others[10]. Prolongation of the PR interval, shortening of the QT interval and lowering of the P wave and the QRS complex, which have been considered as signs of the therapeutic phase[7], were noted in less than 50 per cent of the cats in our experiments. This, however, does not indicate that there was no increase in contractility of the myocardium in the other cats, since it has been established that increased contractility may occur in the absence of any change in the ECG.[3]

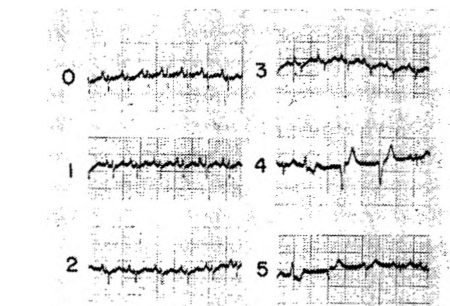

FIG. 4. The ECG of a cat showing an early occurrence of extrasystoles. The cat died after receiving 16 injections of gitalin. Extrasystoles appeared after the fourth injection.

It is not certain why gitalin seems to have a smaller margin of safety in the cat while clinically it has been reported to be superior to the other two glycosides. However, since clinically the end point of toxic doses was the occurrence of any sign or symptom of toxicity, it is possible that digitoxin and acetyldigitoxin are more prone to cause minor toxic signs and symptoms while cardiac arrhythmias are seen more frequently with gitalin administration. It is also possible that gitalin has a greater therapeutic activity than that indicated by the lethal dose; that cardiac glycosides may have a greater margin between the therapeutic and the lethal dose has recently been reported[11].

*Acknowledgement.* The authors wish to acknowledge the valuable technical assistance of Mr. A. F. Peterkin.

## References

1. Cattell and Gold, *J. Pharmacol.*, 1941, **71**, 114.
2. Farah and Maresh, *ibid.*, 1948, **92**, 32.
3. Walton, Leary and Jones, *ibid.*, 1950, **98**, 346.
4. Gruhzit and Farah, *ibid.*, 1953, **108**, 112.
5. Batterman, DeGraff and Rose, *Circulation*, 1952, **5**, 201.
6. Weiss and Steigmann, *Amer. J. med. Sci.*, 1954, **227**, 188.
7. Rothlin and Bircher, *Ergeb. inner. Med. u. Kinderh.*, 1954, **5**, 457.
8. Willems, *Med.*, 1954, **15**, 1163.
9. Walton, *The Cardiac Glycosides, in Pharmacology in Medicine*, Ed. V. A. Dril . McGraw-Hill, New York, 1954.
10. Gold, Gelfand and Hitzig, *J. Pharmacol.*, 1930, **41**, 89.
11. Vick, Kahn and Acheson, *ibid.*, 1957, **119**, 190.
12. Loefler, Essellier and Forster, *Amer. Heart J.*, 1954, **47**, 898.

# THE DISTRIBUTION OF ALKALOIDS IN *RAUWOLFIA CAFFRA* SOND. AND RELATED SPECIES

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In its content of reserpine, *Rauwolfia caffra* root-bark compares favourably with other species. The highest proportion of reserpine is found in the inner phloem whereas the ajmaline group of alkaloids, except for their absence in the cork, are more evenly distributed. The alkaloid content of thin bark exceeds that of thick bark and is very low in the wood of stem and root.

IN a previous communication[1] the structure of the root and stem of *Rauwolfia caffra* Sond. has been described. The root is known to contain rauwolfine[2], ajmaline[3] and reserpine[3,4] but little is known of the distribution of the alkaloids within the plant or of the relative proportions of reserpine to other bases. To clarify this situation, the assays reported below were undertaken and the results compared with similar analyses of related species.

## EXPERIMENTAL

### *Plant Materials and Extraction of Alkaloids*

Four samples of *R. caffra* were investigated. Two were collected by D. B. Fanshawe, Esq., Ndola River, Northern Rhodesia; these comprised thick bark (0·5 cm.) from large roots of unspecified size and smaller whole roots and stems. Another sample was supplied by John Ronaldson Ltd., London and the fourth was collected by S. N. Wimbush, Esq., Conservator of Forests, Northern Nigeria, as *R. welwitschii* Stapf. (now regarded as synonymous with *R. caffra* Sond.). Dr. P. J. Greenway, Nairobi procured the sample of *R. mombasiana* Stapf. and Dr. W. S. S. Ladell the Nigerian *R. vomitoria* Afz. The remaining samples were donated by Riker Laboratories Ltd., England and Riker Laboratories Inc., U.S.A.

A number of root segments of the diameters specified in Table I were selected from each sample, the barks and woods separated and the fractions powdered. For a more detailed analysis of the thick bark of *R. caffra* from Northern Rhodesia, pieces were dissected into five histological portions (Fig. 1) and the corresponding fractions combined to form bulk samples. The cork (1) and the inner functional phloem (5) were easily separated by scraping and the remaining tissues, all containing stone cells, were divided into three portions corresponding approximately to the phelloderm (2) and the largely non-functional outer and middle phloem, (3) and (4).

For the extraction of the alkaloids, 1 g. of powdered sample was intimately mixed with 0·2 g. of calcium hydroxide, moistened with water and allowed to stand overnight. The alkaloids were removed in ether by continuous extraction for 6 hours, *ad hoc* experiments having shown

that under these conditions the extraction of the alkaloids was complete. The residue obtained by removal of the solvent was treated with 2 ml. 1·0N hydrochloric acid and shaken with three separate portions of chloroform. The combined chloroformic extracts were washed successively with 0·13N sodium bicarbonate solution and water and evaporated to dryness under reduced pressure. The residue, protected from light, was used for the determination of reserpine and related alkaloids.

The bases in the acid solution and combined washings were liberated with ammonia, collected in chloroform and washed with water. Evaporation of the solvent furnished a residue containing the more strongly basic alkaloids of the sample, referred to below as the ajmaline group.

Paper chromatography was used to follow the separation process; an amyl alcohol, light petroleum, glacial acetic acid, water mixture (3:1:3:3) proved satisfactory for the development of the chromatograms, which on drying were observed in filtered ultra-violet light. The alkaloids reserpine, rescinnamine, ajmaline and serpentine were used as reference standards. Reserpine, after treatment with acetic acid, gives on irradiation with ultra-violet light, an apple-green fluorescence and similar fluorescent spots of different $R_F$ values were obtained on paper chromatograms from some ajmaline fractions. The small amount of blue fluorescent contaminants in the reserpine fractions could be eliminated in the quantitative assay by the use of a suitable filter and by the enhancement of the reserpine fluorescence as described below. Other authors have also drawn attention to the fluorescent contaminants in the assay of *R. serpentina* root[5-7].

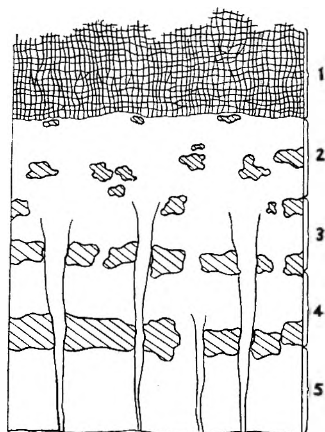


FIG. 1. *Rauwolfia caffra Sond.* General diagram of transverse section of root-bark × 10. 1, cork; 2, phelloderm; 3, outer phloem; 4, middle phloem; 5, inner phloem. Shaded areas of 2, 3 and 4 indicate stone-cell groups.

*Estimation of the Alkaloids*

The assay of the dried reserpine fractions was based on Dechene's method[8] which in the range of 0·4 to 1·8 μg. reserpine per ml. shows an accuracy of +3·2 to −1·0 per cent. Each residue was dissolved in 5N acetic acid and diluted to 100 ml. Several aliquots of each solution, varying from 0·5 ml. for bark samples to 40 ml. for wood samples, were mixed with 3 ml. of 3 per cent hydrogen peroxide solution and the volumes adjusted to 50 ml. with 5N acetic acid. The solutions were heated in a boiling water bath for 45 minutes, cooled to room temperature and the volume readjusted with 5N acetic acid. The fluorescence of the solution was recorded in a modified Hilger fluorimeter using as primary and

secondary filters, Chance OX1 and OG2 filters respectively. The concentration of reserpine in each solution was obtained by reference to a standard curve using those drum readings from aliquots which corresponded to the same, most suitable, region of the curve. The standard curve was prepared by the use of standard reserpine solutions, of the

TABLE I

| Sample* | Alkaloids, per cent** | | | |
|---|---|---|---|---|
| | Reserpine Group | | Ajmaline Group | |
| *Rauwolfia caffra* | | | | |
| Sample 1. N. Rhodesia | | | | |
| Bark 0·5 cm. thick .. | 0·120 ± 0·004 (3) | | 0·303 ± 0·002 (4) | |
| Ditto dissected as Fig. 1 | | | | |
| Cork .. .. .. | 0·009 ± 0·001 (3) | | 0·000 | |
| Phelloderm .. .. | 0·053 ± 0·002 (5) | | 0·344 ± 0·001 (5) | |
| Outer phloem .. .. | 0·153 ± 0·007 (3) | | 0·344 ± 0·001 (6) | |
| Middle phloem .. .. | 0·157 ± 0·003 (3) | | 0·278 ± 0·000 (7) | |
| Inner phloem .. .. | 0·185 ± 0·003 (4) | | 0·277 ± 0·002 (4) | |
| | Bark | Wood | Bark | Wood |
| Sample 2. Bulked roots (2·7 cm.) .. .. .. | 0·550 ± 0·012 (3) | 0·011 ± 0·002 (3) | 0·926 ± 0·002 (3) | 0·009 ± 0·001 (4) |
| Sample 3. N. Rhodesia | | | | |
| Bulked roots (12·0 cm.) | | | | |
| Bark 0·4 cm. thick .. | 0·098 ± 0·009 (3) | 0·008 ± 0·000 (3) | 0·292 ± 0·001 (8) | 0·031 ± 0·002 (4) |
| Stems .. .. .. | † | 0·007 ± 0·001 (3) | trace | trace |
| Sample 4. N. Nigeria (supplied as *R. welwitschii*) | | | | |
| Bulked roots (2·0 cm.) .. | 0·187 ± 0·007 (3) | 0·017 ± 0·001 (3) | 0·413 ± 0·002 (3) | 0·031 ± 0·001 (3) |
| Stem bases (6·0 cm.) .. | 0·005 ± 0·000 (3) | 0·006 ± 0·001 (3) | 0·004 ± 0·000 (3) | trace |
| *R. vomitoria* | | | | |
| Sample 1. Nigeria | | | | |
| Bulked roots (1·0 cm.) .. | 0·410 ± 0·006 (3) | 0·040 ± 0·003 (3) | 0·618 ± 0·003 (4) | 0·041 ± 0·002 (3) |
| ,, ,, (2·0 cm.) .. | 0·420 ± 0·000 (3) | 0·053 ± 0·001 (3) | 0·587 ± 0·001 (4) | 0·030 ± 0·002 (3) |
| Sample 2. Commercial sample | | | | |
| Bulked roots (1·0 cm.) .. | 0·648 ± 0·008 (4) | 0·037 ± 0·001 (3) | 0·704 ± 0·002 (3) | 0·034 ± 0·001 (3) |
| ,, ,, (2·0 cm.) .. | 0·670 ± 0·004 (4) | 0·052 ± 0·002 (2) | 0·621 ± 0·003 (3) | 0·032 ± 0·000 (3) |
| *R. mombasiana*. Kenya | | | | |
| Bulked roots (2·5 cm.) .. | 0·537 ± 0·003 (3) | 0·030 ± 0·005 (3) | 1·181 ± 0·002 (3) | 0·038 ± 0·002 (3) |
| *R. serpentina*. India | | | | |
| Sample 1. | | | | |
| Bulked roots (1·0 cm.) .. | 0·150 ± 0·000 (4) | 0·017 ± 0·002 (3) | 1·875 ± 0·009 (3) | 0·062 ± 0·002 (4) |
| ,, ,, (2·0 cm.) .. | 0·120 ± 0·006 (3) | 0·014 ± 0·001 (3) | 1·613 ± 0·003 (3) | 0·031 ± 0·001 (3) |
| Sample 2. | | | | |
| Bulked roots (1·0 cm.) .. | 0·100 ± 0·006 (3) | 0·027 ± 0·000 (3) | 0·335 ± 0·002 (4) | 0·060 ± 0·000 (3) |
| ,, ,, (2·0 cm.) .. | 0·095 ± 0·005 (2) | 0·019 ± 0·002 (3) | 0·324 ± 0·002 (4) | 0·029 ± 0·001 (3) |
| *R. micrantha* | | | | |
| Bulked roots (1·0 cm.) .. | 0·143 ± 0·007 (3) | 0·032 ± 0·002 (3) | 0·388 ± 0·003 (3) | 0·025 ± 0·001 (3) |
| ,, ,, (2·0 cm.) .. | 0·057 ± 0·003 (3) | 0·019 ± 0·001 (3) | 0·177 ± 0·001 (3) | 0·016 ± 0·000 (3) |
| *R. tetraphylla* (*R. canescens L.*) | | | | |
| Bulked roots (1·0 cm.) .. | 0·160 ± 0·000 (2) | 0·028 ± 0·004 (3) | 0·965 ± 0·006 (3) | 0·039 ± 0·000 (3) |
| ,, ,, (2·0 cm.) .. | 0·163 ± 0·003 (2) | 0·031 ± 0·001 (3) | 1·008 ± 0·008 (3) | 0·037 ± 0·001 (2) |

* Measurements refer to diameter of member except where stated otherwise.
** Arithmetic means ± the standard error with number of assays in parenthesis.
† Assay unreliable—see text.

above concentration range, treated with acetic acid and hydrogen peroxide. Repeated checks of the standard curve throughout the investigation indicated no changes due to instrument variability.

For the determination of the ajmaline group of alkaloids, the solid extract was dissolved in ether and a little chloroform if necessary, and a solution of bromocresol green indicator neutralised to pH 4·5 added. With constant mixing of the two layers, the system was titrated with

0·0025N sulphuric acid to match a prepared end point of pH 4·5. For convenience the alkaloid content was calculated in terms of ajmaline although for some species this may not be the major constituent.

## Results and Discussion

The results of assays are given in Table I. For *R. vomitoria* a total alkaloid content of 1·04 per cent has been recorded, of which 10 per cent is reserpine[9]. Roots of small diameter contain a higher percentage of reserpine than the larger ones[10] but no separate analyses for bark and wood are recorded. The results reported here suggest that with *R. vomitoria*, *R. serpentina* and *R. tetraphylla* the barks of roots of diameter 1 cm. and 2 cm. from any one sample of a species do not differ appreciably in their alkaloid content. Due however, to the relatively higher proportion of wood to bark in the larger roots, the alkaloid content for the 2 cm. whole root would be less than that for the 1 cm. root. As with the other African species *R. vomitoria* and *R. mombasiana*, the root-bark of *R. caffra* contains commercially workable quantities of reserpine and, with barks varying considerably in thickness, a relation between size and alkaloidal content is evident. Should this species be used commercially, the root-wood, which contains little alkaloid, could be rejected at the time of collection. The relative proportions of alkaloids in the root-wood and root-bark appear in most instances to be similar for all the species examined.

With the sample of *R. caffra* bark examined in detail, the alkaloid mixture is not evenly distributed throughout the root-bark (Fig. 1). The cork contains practically no alkaloids. Reserpine is found in highest concentration in the inner phloem with much less in the phelloderm, whereas the ajmaline group of alkaloids are more uniformly distributed with highest proportions in the phelloderm and outer phloem.

It was not possible to make a successful determination of the reserpine content of the stem-bark owing to the presence of an interfering fluorescent principle in the appropriate fraction. Paper chromatograms indicated the possible presence of a small quantity of reserpine and in view of references[9,11] to the use of the stem-bark by native tribes an attempt was made to isolate the weakly basic constituents from 50 g. of bark originating from Northern Rhodesia. No crystalline alkaloid could be isolated; a similar negative result had previously been reported by Schüler and Warren with material from Natal[3].

## References

1. Court, Evans and Trease, *J. Pharm. Pharmacol.*, 1957, **9**, 237.
2. Koepfli, *J. Amer. chem. Soc.*, 1932, **54**, 2412.
3. Schüler and Warren, *J. chem. Soc.*, 1956, 215.
4. Aleer, Weston and Howe, *Chem. and Ind.*, 1956, 1387.
5. Banerjee and Chatterjee, *Bull. Calcutta School Trop. Med.*, 1955, **3**, 164.
6. Sen, Banerjee and Chatterjee, *ibid.*, 1957, **5**, 66.
7. Banerjee, Chatterjee and Häusler, *J. Pharm. Pharmacol.*, 1957, **9**, 120.
8. Dechene, *J. Amer. pharm. Ass. Sci. Ed.*, 1955, **44**, 657.
9. Dubois, *Bull. agr. Congo Belge*, 1955, **46**, 566.
10. Monseur, *J. Pharm. Belg.*, 1957, **12**, 39.
11. Feuell, *Colon. Pl. Anim. Prod.*, 1955, **5**, 13.

# THE PHYSICAL PROPERTIES OF LYSOLECITHIN AND ITS SOLS

## PART I.—SOLUBILITIES, SURFACE AND INTERFACIAL TENSIONS

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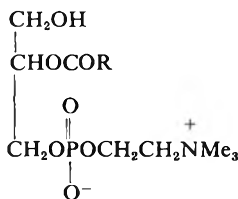
The solubilities of lysolecithin in water and some organic solvents have been determined and the effect of lysolecithin at air-water and chloroform-water interfaces examined. The results indicate that lysolecithin has marked surface-active properties and that a critical micelle concentration occurs in the aqueous sols in the range 1 to $2 \times 10^{-3}$ per cent weight/volume. The effects of acid, alkali and mono- and di-valent cations on the lowering of the interfacial tensions was found to be small. Lysolecithin had little or no lowering effect on the surface tensions of ethanol and chloroform.

THIS work is a continuation of the study of the colloidal properties of lecithin and lysolecithin[1–5], in relation to the structure of cell membranes. A molecule of lysolecithin can be regarded as having two distinctly different regions, a long hydrocarbon chain of non-polar character which is lipophilic and a phosphoric acid-choline radical, polar in nature, which is hydrophilic.




Zwitter-ion structure of lysolecithin
(R is a saturated hydrocarbon chain, principally $C_{13}$ to $C_{17}$)

The molecule, therefore, possesses an amphipathic character (Hartley[6]) and can be expected to show surface-active properties comparable with soaps. The surface activity of lysolecithin was investigated at air-water and chloroform-water interfaces and the work extended to examine the effects of acid and alkali and mono- and di-valent salts on these interfaces. The effects of lysolecithin on the surface tension of ethanol and chloroform were also determined.

The surface tension measurements were carried out using the ring (dynamic)[7] and Wilhelmy plate (static)[8] methods. The ring method only was used for the experiments on interfacial tension.

Some measurements were made to obtain information on the solubility of lysolecithin in water and various organic solvents.

## EXPERIMENTAL

### *Preparation of Lysolecithin*

Mixed phosphatides were prepared by separating the yolks of twelve eggs, extracting them repeatedly with acetone until a white powder was

obtained, and this was further extracted with successive portions (100, 300, 100 ml.) of ethanol. The ethanolic solution of the crude lecithin was then shaken with successive portions of alumina powder until the solution was free from amino-nitrogen phosphatides. About 6 g. of lecithin was obtained at this stage and stored under nitrogen-saturated absolute ethanol at − 5°.

Lysolecithin was prepared from a 5 g. sample of lecithin by Saunders modification[5] of the method of Hanahan[9] using Russell viper venom.

TABLE I

SOLUBILITY OF LYSOLECTHIN IN SOME ORGANIC SOLVENTS

| Solvent | Solubility in g./100 ml. solution 25° C. | 40° C. |
|---|---|---|
| Chloroform . . . . | 27·830 | 32·400 |
| Methanol . . . . | 14·600 | 19·130 |
| Ethanol . . . . . . | 3·870 | 10·690 |
| Pyridine . . . . | 0·330 | 1·030 |
| Acetone . . . . . . | 0·022 | 0·085 |
| Diethyl ether . . . . | 0·002 | 0·076 |
| Light petroleum 60°–80° | 0·023 | 0·062 |
| Benzene . . . . . . | 0·012 | 0·057 |

The yield was about 2 g. of a white solid which had a nitrogen content of 2·72 per cent, a phosphorus content of 5·98 per cent, an iodine value of 5·5 and $[\alpha]_D^{21} = + 2{\cdot}26°$ (5·457 g. in 100 g. ethanol). The mean molecular weight calculated from the nitrogen and phosphorus contents was 516. Calculated values for nitrogen and phosphorus in hydrated lysolecithin when the fatty acid is palmitic, molecular weight 513, are 2·72 and 6·04

TABLE II

VARIATION OF SURFACE TENSION OF LYSOLECITHIN SOLS WITH TIME

| | Static method | | Dynamic method |
|---|---|---|---|
| Time | Concn 0·0104 per cent w/v lysolecithin γ20° | Concn 0·00104 per cent w/v lysolecithin γ20° | Concn 1·076 per cent w/v lysolecithin γ20° |
| 0 min. | 39·67 | 43·95 | 37·93 |
| 2 ,, | 39·63 | 43·94 | 37·50 |
| 5 ,, | 39·62 | no change | 37·26 |
| 10 ,, | no change | ,, | 37·17 |
| 20 ,, | ,, | ,, | 37·01 |
| 30 ,, | ,, | ,, | 36·88 |
| 60 ,, | ,, | ,, | 36·86 |
| 120 ,, | ,, | ,, | 36·68 |
| Agitation | — | — | 37·91 |
| 140 ,, | — | — | 37·86 |
| 150 ,, | — | — | 37·57 |
| 15 hours | 39·60 | 43·55 | — |

respectively. The low iodine value obtained with the highly specific catalyst lecithinase-A indicates the almost complete absence of unsaturated fatty acid groups in the lysolecithin.

In some work on the hydrolysis of lecithin, cobra venom preserved in a cork-stoppered bottle for 30 years was found to possess some activity, 1·25 g. of lysolecithin being obtained from 5 g. of lecithin by a method similar to the above[5]. With this catalyst the hydrolysate formed a gel and not the white precipitate given by the Russell viper venom.

*Preparation of Sols*

Pure aqueous sols of lysolecithin were prepared in distilled water and passed through an ion exchange column containing a mixture of Amberlite resins IR-120 and IRA-400. Electrical conductivity measurements showed that any traces of ions present as impurities had been removed. The column was then washed with distilled water and the combined

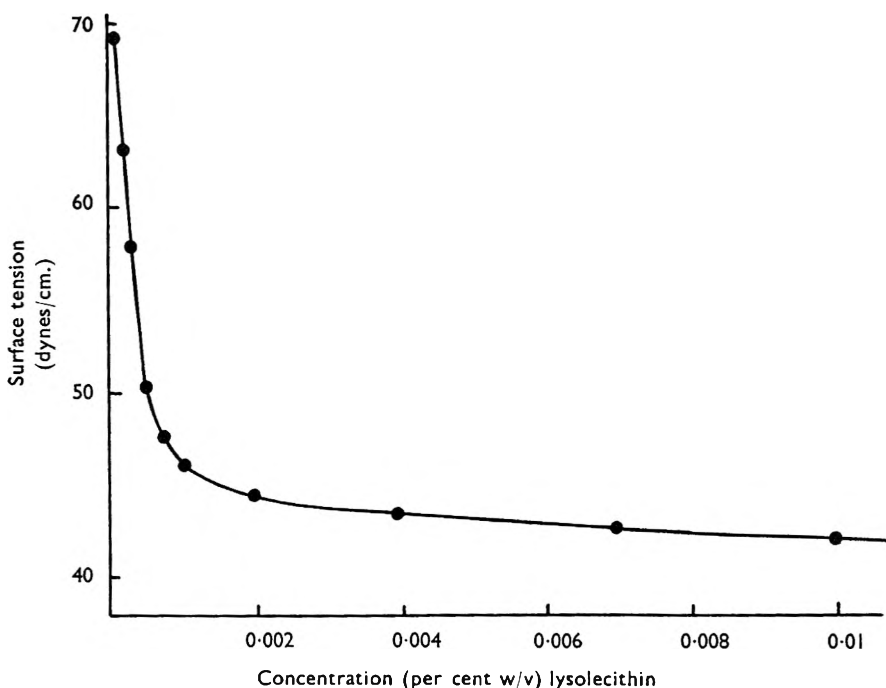



FIG. 1. Variation of surface tension of water (dynamic method) with concentration of lysolecithin. Temperature 20°.

effluent made up to volume to give a concentration of approximately 1·0 per cent w/v. Measured quantities of this concentration were diluted as required.

Solutions of the electrolytes were made with Analar materials and measured quantities mixed with 0·1 per cent w/v lysolecithin sol, shaken and made up to volume. All the solutions were optically clear.

*Specific Rotation*

Specific rotations of lysolecithin in absolute ethanol were made with a Bellingham and Stanley polarimeter.

*Solubility Determination*

The solubility of lysolecithin in a number of solvents was determined at 25° and 40°. The approach to equilibrium was accomplished from undersaturation by adding the solute to the solvent at the temperature

of the experiment. Equilibrium from a supersaturated solution was obtained by shaking the solute and solvent at a slightly higher temperature and allowing to cool to the required temperature. The dissolution was made in a Microid Flask shaking machine operating at a rate sufficient to ensure solution without the production of foam, for a period of four hours. 50 ml. pyrex glass tubes, fitted with ground-glass stoppers, each containing 25 ml. of solution were then allowed to stand upright overnight in a water thermostat, controlled to $\pm$ 0·05°, to enable excess solid to settle. Four tubes were taken for each solvent, two used for undersaturation and two for supersaturation.

For analysis of the saturated solutions, separation was by rapid transfer to the weighing vessels, with an Agla microsyringe preheated to 2° or 3° above the temperature of the solutions. These weighing vessels were 1 ml. rimless beakers with small watch glass covers. Samples of 0·5 ml. were analysed by slow evaporation of the volatile solvents, drying *in vacuo* for 12 hours at 30° and weighing the residue to constant weight. A possible error to be overcome in the determination of solubility was the taking up of moisture by the hygroscopic lysolecithin. Using a pre-set balance no difficulty was found in obtaining for each solvent eight residues which showed close agreement.

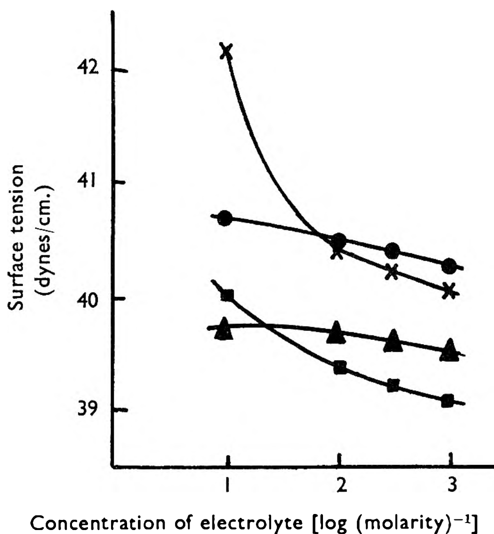



FIG. 2. Effect of various electrolytes on the surface tension of lysolecithin sols. Temperature 20°. Concn. 0·0152 per cent w/v lysolecithin. ▲ HCl; ■ NaOH. Concn. 0·00985 per cent w/v lysolecithin. ● NaCl; × $CaCl_2$.

All organic solvents were purified according to Vogel[10].

*Surface and Interfacial Tension Apparatus*

The solutions for surface tension measurements were contained in a circular pyrex dish 15 cm. diameter and 3 cm. deep, immersed in a water thermostat controlled to $\pm$ 0·05°. The plate and ring suspensions were enclosed within a glass cylinder to prevent disturbances due to the movements of air.

In both the Wilhelmy plate method and the ring method a Webb chainomatic balance reading accurately to $10^{-4}$ g. adapted to the general assembly was used; this was constructed on the same pattern as that of Harkins and Jordan[7]. It stood on a platform which could be moved up and down

slowly and smoothly by means of a rack and pinion mechanism. In the Wilhelmy method a platinum plate was used in preference to glass since it was found to give reproducible results and could be more easily cleaned. The plate, of dimensions 3·500 × 2·602 × 0·0342 cm., was supported from the balance arm by thin chrome-nickel wire. In the ring method a platinum ring of mean diameter 1·765 cm. was suspended by nylon thread.

TABLE III

EFFECTS OF LYSOLECITHIN ON THE SURFACE TENSION OF ETHANOL AND CHLOROFORM

| Ethanol | | Chloroform | |
|---|---|---|---|
| Concn per cent w/v lysolecithin | $\gamma 20^{\circ}$ | Concn per cent w/v lysolecithin | $\gamma 20^{\circ}$ |
| 0·0 | 22·26 | 0·0 | 27·19 |
| 0·985 | 22·17 | 0·985 | 27·19 |
| 0·0985 | 22·18 | 0·0985 | no change |
| 0·00935 | 22·19 | 0·00985 | no change |
| 0·000985 | 22·26 | | |

To ensure that the plate and ring hung horizontally, they were levelled above a metal table which was mirror finished on top.

Chloroform was chosen for the work on interfacial tension.

## RESULTS

### *Solubility*

Lysolecithin dissolved in water to give a perfectly clear solution. At a concentration of 36 per cent by weight the solution became slightly viscous, the viscosity increasing with concentration until a thick fluid, still quite clear, was obtained at 50 per cent. At no time throughout the concentration range was a saturation point reached. The solubility of lysolecithin in some organic solvents is shown in Table I.

### *Surface Tension*

The effects of lysolecithin on the surface tension of aqueous solutions are shown in Figures 1, 2 and Table II; of non-aqueous solutions in Table III.

### *Interfacial Tension*

The interfacial tension effects of lysolecithin on a chloroform-water system are shown in Figures 3 and 4.

## DISCUSSION

### *Surface Tension*

The surface tension of water decreased with increasing concentration of lysolecithin and indicated positive adsorption at the surface. A considerable lowering effect was found at concentrations of less than 0·001 per cent w/v and at this value the surface tension was lowered from 72·67 dyne/cm. to 45·35 dyne/cm., while increasing the concentration of lysolecithin to 1 per cent lowered the surface tension of water to 37·6 dyne/cm.

It is probable that at low concentrations the lysolecithin molecules are present in water as single molecules or small micelles to which the surface activity can be attributed. When the concentration of lysolecithin reached the range 1 to 2 $\times$ $10^{-3}$ per cent w/v its lowering effect on the surface tension of water became noticeably less and it seems likely that this change in behaviour is due to the commencement of formation of

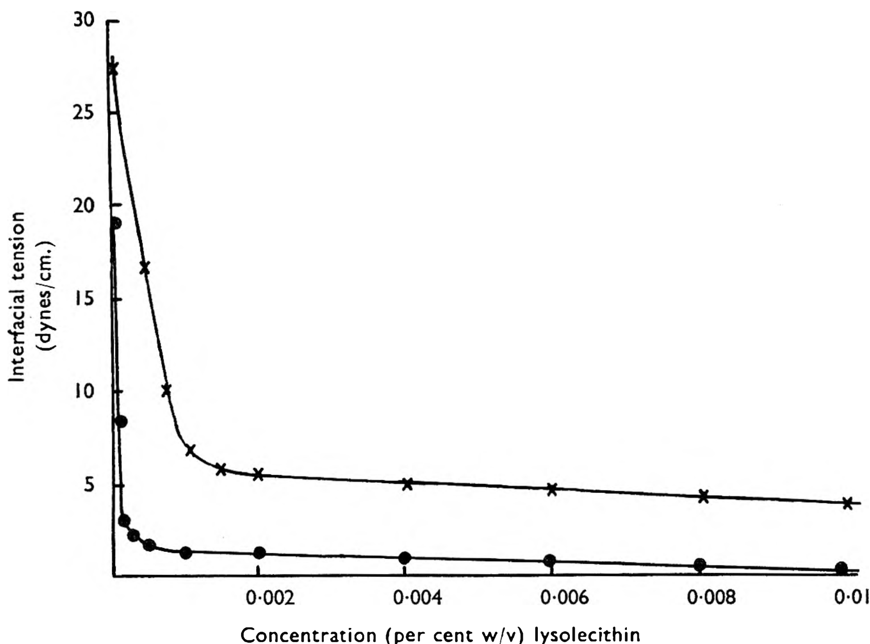



FIG. 3. Variation of interfacial tension of a chloroform-water system with concentration of lysolecithin. Temperature 20°.
× Lysolecithin in water layer; ● Lysolecithin in chloroform layer.

much larger micelles within the solution. Saunders and Thomas[11] have shown in their work on the diffusion of lysolecithin in aqueous media that such larger micelles do exist at higher concentrations (1 to 2 per cent w/v), consisting of aggregates of about 270 single molecules.

The change in surface tension with time at the two concentrations measured by the static method (Table II) showed an initial slight lowering within 5 minutes perhaps due to diffusion of molecules and orientation at the surface layer. Thereafter a further 15 hours showed a very small change whilst equilibrium was being established between the body of the sol and the surface layers. This could be regarded as further evidence for the presence of large particles which are very soluble and remain in the bulk of the sol showing no tendency to migrate to the surface.

Within its limitations the dynamic method gave surface tension-time results (Table II) which showed more clearly the progress towards equilibrium between the surface layer and the bulk of the sol. The surface tension decreased by 0·67 dyne/cm. in the first 5 minutes, followed

by a less rapid fall of 0·58 dyne/cm. in the next 115 minutes. Agitation of the sol then caused restoration of equality of concentration within the system and the surface tension regained its original value, which was again followed by a decrease with time following a similar pattern.

The effects of acid, alkali, sodium and calcium chlorides on the surface tension of a sol of concentration 0·00985 per cent w/v were quite small (Fig. 2). A reduction in the surface activity of the lysolecithin with increasing alkalinity was shown, but in acid conditions there was no change beyond an initial fall. A 0·1 molar calcium chloride solution reduced the surface tension lowering by approximately 7·5 per cent. The stability of lysolecithin sols to salts has been examined by Saunders[5] who found that sodium and calcium chlorides had no precipitating effect on the sols within the concentration range used by the present authors. The effect of these salts may, therefore, have caused interionic forces to predominate at the surface resulting in desorption of lysolecithin.

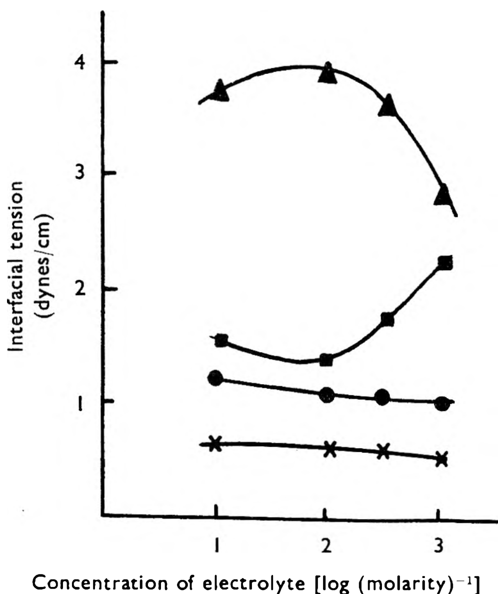



FIG. 4. Effect of various electrolytes on the chloroform (containing 0·01 per cent w/v lysolecithin)–water interface of tension 0·6 dyne/cm. Temperature 20°.
▲ HCl; ■ NaOH; ● NaCl; × $CaCl_2$.

No lowering of the surface tension of chloroform by lysolecithin within the concentration range 0·1 per cent to 0·001 per cent w/v took place. The surface tension lowering of ethanol by a 0·1 per cent w/v lysolecithin solution was only 0·09 dyne/cm. Pure lysolecithin is quite soluble in chloroform and ethanol and there appears to be no tendency to repel either polar or non-polar regions of the molecule from the solvents, and consequently no surface layer is formed.

*Interfacial Tension*

The general pattern of the lowering of the interfacial tension of the chloroform-water system with increasing concentration of lysolecithin (Fig. 3) is similar to that at the air-water interface (Fig. 1). This is not necessarily to be expected, since both hydrophilic and lipophilic characteristics come into play at the chloroform-water interface.

The greatest lowering effect took place with a concentration of $10^{-4}$ per cent w/v lysolecithin dissolved in the chloroform layer when the

interfacial tension of 32·73 dyne/cm. decreased to 2·91 dyne/cm. (Fig. 3). This was further lowered to less than 1 dyne/cm. by increasing the concentration to $10^{-2}$ per cent w/v which enabled the two liquids to be readily emulsified with the slightest mixing. The final lowering to 0·35 dyne/cm. at a concentration of 0·1 per cent w/v lysolecithin in chloroform was 3 dyne/cm. more than when lysolecithin was dissolved in the aqueous phase.

The greater surface activity shown when lysolecithin was dissolved in the chloroform layer may have been due to the presence of smaller micelles than those in the aqueous layer. When lysolecithin was dissolved in the aqueous layer the interfacial tension between the concentration range 1 to $2 \times 10^{-3}$ per cent w/v became noticeably less in a way similar to that shown at the air-water interface. Aggregation of single molecules or small micelles is again probably taking place in the aqueous phase within this concentration range.

The effects of acid, alkali and sodium chloride in the aqueous phase (Fig. 4) were small and comparable to those at the air-water interface; the effect of calcium chloride, however, was less marked at the chloroform-water interface.

*Acknowledgements.* The authors thank Dr. A. C. White of the Wellcome Research Laboratories for the gift of Russell viper venom and Mr. P. Barden for his help in adapting the chainomatic balance.

## References

1. Saunders, *J. chem. Soc.*, 1953, 519.
2. Saunders, *ibid.*, 1953, 1310.
3. Elworthy and Saunders, *ibid.*, 1955, 1166.
4. Elworthy and Saunders, *ibid.*, 1957, 330.
5. Saunders, *J. Pharm. Pharmacol.*, 1957, **9**, 834.
6. Hartley, *Aqueous solutions of Paraffin-chain Salts*, Hermann et Cie, Paris, 1936.
7. Harkins and Jordan, *J. Amer. chem. Soc.*, 1930, **52**, 1751.
8. Harkins and Anderson, *ibid.*, 1937, **59**, 2189.
9. Hanahan, Rodbell and Turner, *J. biol. Chem.*, 1954, **206**, 431.
10. Vogel, *Practical Organic Chemistry*, 3rd edit. Longman, 19+**6**.
11. Saunders and Thomas, *J. chem. Soc.*, 1958, 483.

# ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

## CHEMISTRY

### ANALYTICAL

**Codeine, Narcotine and Thebaine, Determination of, in Poppy Capsules.** J. Holubek, S. Kudrnác and M. Novák. (*Die Pharmazie*, 1958, **13**, 95.) In this method, codeine and narcotine are separated from the other opium alkaloids by paper chromatography. Codeine is then determined colorimetrically by oxidation with potassium permanganate, removal of excess with ferrous ammonium sulphate, treatment with alkali to remove the iron, and colour development with equal parts of diazo reagents I and II of the *Deutsche Artzeneibuch*, 6th edition. It is shown that narcine, which could not be separated from codeine by paper chromatography, did not interfere. In amounts between 50 and 200 $\mu$g., the results are within $\pm 2$ per cent. Narcotine is determined after elution from the paper by hydrolysis to cotarnine with dilute sulphuric acid, followed by polarography in alkaline solution. In amounts between 50 and 200 $\mu$g., the results are within $\pm 4$ per cent. Full details are given for the preparation and purification of the extract, and the results are tested by the addition of known amounts of codeine and narcotine to assayed extracts and redetermination. Very good agreement is obtained. The assay of thebaine after elution from the filter paper was however not satisfactory. The method given is a direct colorimetric one based on hydrolysis in acid solution to codeinone and coupling with diazotised sulphanilic acid. Excess of codeine, narcotine and papavarine did not interfere. The method is suitable for amounts up to 100 $\mu$g. of thebaine. The soundness of this method was again proved by addition of known amounts of thebaine to assayed extracts and reassay.

D. B. C.

**Cycloserine and Isoniazid, Spectrometric Determination of, in Pharmaceutical Preparations.** J. M. Woodside, I. Piper and J. B. Leary. (*J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 729.) Pharmaceutical preparations of cycloserine may be assayed by measurement of the ultra-violet absorption of a suitable solution in 0·1 N hydrochloric acid at 219 m$\mu$, calculating the cycloserine content from the datum $A_{219m\mu} = 0{\cdot}341$ for a solution containing 10 $\mu$g. of cycloserine per ml. When isoniazid is present in addition to cycloserine, a further measurement is made at 272 m$\mu$. Cycloserine does not absorb at this wavelength, and the isoniazid content may be calculated directly. To allow for the absorption at 219 m$\mu$ due to isoniazid, the absorption at 272 m$\mu$, multiplied by 1·13 is subtracted from the absorption at 219 m$\mu$ before calculating the cycloserine content of the sample.

G. B.

***n*-Butanol as a Standard of Haemolytic Index.** J. Vandeputte-Poma and R. Ruyssen. (*Pharm. Weekbl.*, 1958, **93**, 94.) As a standard of comparison for the determination of haemolytic index, *n*-butanol is preferable to other substances such as saponins, because it is less variable in composition. The authors report a detailed study of haemolysis by *n*-butanol with a view to its use as a standard. A method of assay based on comparison of the concentration of sample and standard causing 50 per cent haemolysis of a suspension of erythrocytes is recommended.

G. B.

# BIOCHEMISTRY

## GENERAL BIOCHEMISTRY

**2-Methoxyoestrone, a new Metabolite of Oestradiol-17β in Man.** S. Kraychy and T. F. Gallagher. (*J. biol. Chem.*, 1957, **229**, 519.) After the administration of oestradiol-17β-16-$^{14}$C to human subjects, 2-methoxyoestrone was isolated from the urine. The structure of this compound was established by synthesis. It is suggested that this steroid, a methylated *o*-hydroquinone, is a normal metabolite of the oestrogenic hormone. The significance of this is that oxidation of the aromatic ring is achieved under *in vivo* conditions and probably this is a preliminary stage to fission of the carbon skeleton. In this respect the reaction would be similar to the *in vivo* formation of 3:4-dihydroxyphenylalanine from tyrosine. It is also of interest that this is a reaction involving the methylation of oxygen, perhaps similar to the *O*-methylation *in vivo* of 3:4-dihydroxymandelic acid, a metabolite of noradrenaline in man. That two such aromatic hormones are similarly altered emphasises the endocrine significance of this type of biochemical transformation. This work suggests that the metabolism of oestrone or oestradiol is accomplished in two separate stages; firstly oxidation and subsequently methylation. M. M.

**Nalorphine, Inhibitory Action of, on the Enzymatic *N*-Demethylation of Narcotic Drugs.** J. Axelrod and J. Cochin. (*J. Pharmacol.*, 1957, **121**, 107.) It has been shown that a variety of narcotic drugs are *N*-demethylated by an enzyme system found in mammalian liver microsomes. Since nalorphine blocks the pharmacological action of the same compounds that are attacked by this enzyme system a study is made to determine whether or not its inhibitory action extends to the process of *N*-demethylation. The degree of *N*-demethylation in rat liver enzyme preparations incubated with morphine alone and combinations of morphine with varying amounts of nalorphine, was determined. Marked inhibition of morphine demethylation, directly related to the nalorphine concentration, was observed. It appears that this inhibition is non-competitive but it is possible that it is a slow pseudo-irreversible inhibition mimicking non-competitive inhibition but occurring at the same site. Studying a series of narcotic drugs, it was found that, with the exception of cocaine, the *N*-demethylation of such substances was inhibited by nalorphine. There was greater inhibition of the demethylation in compounds in which the methyl group is attached to the nitrogen atom of the piperidine ring. The effect of nalorphine on other pathways of drug metabolism was also investigated. Nalorphine blocked the enzymatic *O*-demethylation of codeine but had no effect on the *O*-demethylation of *p*-methoxybenzonitrile. The enzymatic de-esterification of pethidine was not blocked by nalorphine and the side-chain oxidation of hexobarbitone was inhibited only slightly. Studying analogues of nalorphine, it was found that, with the exception of *N*-propyl*nor*morphine, *N*-substituted *nor*morphine derivatives with a 3-carbon chain linked to the nitrogen atom are the most potent inhibitors. Nalorphine was found to be dealkylated to *nor*morphine by enzymes in the rat liver microsomes when incubated with the soluble supernatant fraction of rat liver, triphosphopyridine nucleotide and nicotinamide. The rate of dealkylation of nalorphine was more than twice that of morphine.
M. M.

# BIOCHEMICAL ANALYSIS

**Acetylcholinesterase Activity, Colorimetric Determination of.** D. N. Kramer and R. M. Gamson. (*Analyt. Chem.*, 1958, **30**, 251.) This depends on hydrolysis by the enzyme at pH 8·0 of indophenyl acetate (*N*-4′-acetoxyphenyl)-*p*-quinone imine which is reddish yellow to the blue-purple *p*-quinone imine *N*-4′-phenoxide ion. A blank determination is performed and the absorbance of the hydrolysis product is measured at 625 m$\mu$ after a definite time, normally 15 minutes but may be extended to two hours for weak solutions of the enzyme. The method is precise and reproducible in the range of 25 to 150 units of acetylcholinesterase. The effect of variables such as pH, substrate concentration, time, stability of reagents and the effects of alcohol, albumin and hydrolysis product is discussed. D. B. C.

**Parathion, Detection and Quantitative Determination of, in Biological Materials.** K. Erne. (*Acta pharm. tox., Kbh.*, 1958, **14**, 173.) A specific and sensitive method is described for the identification and quantitative determination of parathion and *p*-nitrophenol in biological material. Acidify 10 g. of the homogenised sample with 1 ml. of 5 M sulphuric acid, triturate with 20 g. of Hyflo Supercel and extract with benzene in a Soxhlet for four hours. Concentrate in a current of air to 25 ml., filter and chromatograph the solution on an alumina column. Elute the parathion with ether and the *p*-nitrophenol with methanol. The parathion is analysed by paper chromatography developed with *iso*-octane saturated with dimethyl sulphoxide. It is identified by a yellow band at $R_F$ 0·7 formed by spraying the dried paper with 0·5 M ethanolic potassium hydroxide followed by heating at 100° for 5 minutes. A quantitative determination is made by excising this area from another strip of paper prepared in the same way. This is extracted by suspending the paper in a condenser and refluxing into a small flask with ethanol. Add through the condenser 4 drops of 5 M hydrochloric acid, 3 ml. of water and one granule of zinc and reflux for ten minutes. Transfer the liquid to a 10 ml. volumetric flask, and add at four minute intervals 4 drops of 0·25 per cent sodium nitrite, 2·5 per cent ammonium sulphamate and 1 per cent naphthyl ethylenediamine solutions. Add water to 10 ml. and thirty minutes later read the extinction at 555 m$\mu$ against a blank. Compare with a standard parathion calibration curve prepared the same way. The *p*-nitrophenol is determined in the methanol eluate evaporated to 10 ml. Add 1 ml. of 5 M sodium hydroxide, 50 ml. of water and extract the lipids with ether. Acidify, extract with ether, wash the extracts with 25 per cent sodium sulphate, dry with anhydrous sodium sulphate, wash with ether and adjust the volume to 0·4 ml. with methanol. Carry out paper chromatography developed with *n*-butanol saturated with 5 M ammonia. Air-dry the paper, and a yellow band at $R_F$ 0·5 indicates the presence of *p*-nitrophenol. For quantitative estimation eluate the excised yellow band by refluxing with methanol as before. To the solution add 1 drop of 5 M sodium hydroxide and methanol to 10 ml. Read the extinction in the 350 to 450 m$\mu$ region. Compare with a standard curve prepared in the same way. G. F. S.

**Tolbutamide, Determination of, in Serum.** E. Bladh and Å. Nordén. (*Acta pharm. tox. Kbh.*, 1958, **14**, 188.) A method is described for determining tolbutamide based on Forist's method of chloroform extraction and ultra-violet measurement at 228 m$\mu$. Mix one ml. of serum with 1 ml. of water and 1 ml. of 6 N hydrochloric acid. Shake for 15 minutes with 20 ml. of chloroform, separate and wash with 2 ml. of chloroform. Pass the chloroform solutions containing the tolbutamide through a chromatography tube packed

with calcium carbonate or sodium bicarbonate and evaporate under reduced pressure at 30° in a flask. Add 10 ml. of absolute ethanol to dissolve the residue and measure the absorbancy of the solution in a spectrophotometer at 228 mμ. A serum sample without the tolbutamide is carried through the same procedure as the blank. The serum concentration of tolbutamide is determined from a standard curve, and the accuracy of the method is ±0·6 mg. per cent. The method may be improved by buffering the plasma to pH 4·5 to 4·8 before chloroform extraction. Recently Spingler (*Klin. Wschr.*, 1957, **35**, 533) has introduced a colorimetric method, which makes use of the formation of butylamide when tolbutamide in an inert solution medium is exposed to increased temperature. Butylamine gives a yellow colour with dinitrochlorbenzene or dinitrofluorbenzene. G. F. S.

**Vitamin $B_{12}$ in Natural Materials of low Potency, Estimation of.** J. M. McLaughlan, C. G. Rogers, E. J. Middleton and J. A. Campbell. (*Canad. J. Biochem. Physiol.*, 1958, **36**, 195.) Existing methods for the estimation of vitamin $B_{12}$ in low potency materials frequently lack specificity and often are subject to considerable variation. The *Lactobacillus leichmanii* assay for vitamin $B_{12}$ does not give reliable results in the presence of desoxyribosides and certain of the vitamin $B_{12}$-like factors to which *L. leichmanii* responds. A radioactive tracer assay which is specific for vitamin $B_{12}$, and which has been adopted by the U.S.P., is not applicable to materials of low potency because of the necessity of measuring the vitamin spectrophotometrically. This paper describes the modification of the method so that it is suitable for low potency materials. The purification procedure is effective in removing pseudovitamin $B_{12}$ and factor A but is ineffective in removing vitamin $B_{12}$ III. A correction factor is used in place of $^{60}$Co-labelled vitamin $B_{12}$ to adjust for loss during purification. This purification does not affect the estimates of vitamin $B_{12}$ activity of milk, plasma, chick mash, or fish meal. True vitamin $B_{12}$ activity is apparently responsible for 50 per cent of the total vitamin $B_{12}$ activity of dried cattle faeces and for less than 10 per cent of the total activity of yeast activity. The coefficient of variation of the modified method was 8·7 per cent based on 20 estimates of potency. M. M.

## PHARMACY

**Adrenaline and Noradrenaline, The Stability of Injections of.** J. Mørch. (*Pharm. Weekbl.*, 1958, **93**, 141.) Decomposition was assessed by determinations of biological activity in rats. The injection solutions of the Danish Pharmacopoeia were investigated; they contained 0·1 per cent of adrenaline with hydrochloric acid or 0·01 per cent of noradrenaline as bitartrate, and the solutions were prepared using water redistilled in glass, containing 0·05 per cent of sodium metabisulphite (pyrosulphite). The solutions before autoclaving had a pH of 3 or 4, which decreased to 2·9 or 3·4 owing to the oxidation of metabisulphite to sulphuric acid during sterilisation. Solutions of adrenaline lost about 4 per cent of their activity during autoclaving at 120° for 20 minutes, and noradrenaline solutions lost only 2 per cent. All solutions remained colourless after this treatment. On heating at 100° for 27 hours, the solutions were little affected by the presence of 2 μg. of copper ion per ml., but considerable decomposition occurred in the presence of 20 μg./ml. During storage at 100°, oxidation occurred slowly at first, the observed loss in activity being mainly due to racemisation. Later, as the metabisulphite was destroyed, the oxidation

took place more rapidly, and finally more slowly as the oxygen in the container was used up. The presence of metabisulphite prevented discoloration, but even when it was added in excess compared with the oxygen content of the ampoule it did not completely prevent the decomposition of adrenaline or noradrenaline.

G. B.

**Adrenaline, Oxidation of, *In Vitro*, by Copper in the Presence of Plasma and Plasma Fractions.** P. Varène. (*Bull. Soc. Chim. Biol.*, 1957, **39**, 1473.) The decomposition of adrenaline in solution in the presence of copper ions was assessed biologically and colorimetrically as described previously (see abstract *J. Pharm. Pharmacol.*, 1958, **10**, 332). The reaction was considerably retarded by the addition of heparinised plasma to the solution. The albumin fraction had the greatest retarding effect, and protected the adrenaline as effectively as a quantity of plasma containing the same weight of proteins. The globulins were inactive, and the dialysable fraction showed only a slight effect, which was attributed to its amino acid content. There appears to be no simple protective mechanism, but if it is assumed that the catalyst can become partly attached to the protein, it seems that the adrenaline must also form bonds with proteins.

G. B.

**Bacteriostatics For Parenteral Injections of Procaine Penicillin.** C. L. Sargent. (*Pharm. Weekbl.*, 1958, **93**, 81.) Solutions containing 50,000 to 500,000 units/ml. prepared from penicillin admixed with 4 per cent of sodium citrate were shown to retain their original potency for at least 8 days when stored at 4°. At 25°, solutions containing 100,000 units/ml. were stable for 4 days if made from penicillin-citrate mixture containing 4 or 5 per cent of sodium citrate, but not if made from a mixture containing 3 per cent of citrate. Stronger solutions, containing 500,000 units/ml. were less stable and did not retain their potency for 4 days at 25° when made from a mixture containing 4 or 5 per cent of citrate. Deterioration of the solutions was not invariably accompanied by obvious physical change. An attempt was made to find an antiseptic which would confer on procaine penicillin suspensions about the same bacteriostatic power as 0·5 per cent phenol solution. Phenylmercuric nitrate 0·001 per cent and propyl hydroxybenzoate 0·02 per cent with methyl hydroxybenzoate 0·2 per cent were rejected as ineffective; moreover in the presence of the hydroxybenzoates a precipitate tended to form in 5 to 7 days. Benzyl alcohol 1·5 per cent and phenol 0·5 per cent showed a satisfactory bacteriostatic effect, but rendered the suspensions viscous after a few days' storage and afterwards caused the development of a yellow colour. Cetrimide 0·01 per cent was the most satisfactory bacteriostatic agent examined, but adsorption onto the medicament occurred, and the whole suspension had a greater bactericidal effect than the supernatant liquid. Samples of cetrimide from several sources varied in bactericidal potency, and it is suggested that a minimum amount should be specified.

G. B.

**Cod Livers, Investigation of the Oil Obtained by a Freezing Procedure from.** S. Erbe, (*Arch. Pharm.*, 1958, **28**, 1.) The following freezing process for the extraction of the liver oil is said to give a product of higher vitamin activity and pleasanter taste than those obtained using superheated steam:—The fresh livers are slowly frozen so that large ice crystals form in the cells. Temperatures down to −50° are used. Rapid freezing causes the formation of small crystals so that the cells are not completely disrupted in the subsequent grinding process, resulting in loss of oil. After fine mincing and thawing in the absence of air as far as possible, the oil is separated by centrifuging. The taste of such an

oil is only weakly fishy and the oil will keep four to five months at 3–5° before a slight alteration in taste is noticed. It is important, however, not to allow the livers to remain in a frozen condition for too long, since even after a few days the acid value rises appreciably, probably due to the activity of tissue lipases which remain active at temperatures as low as −25° to −30°. D. B. C.

**Solutions Varying From the Normal Osmotic Pressure and Hydrogen Ion Concentration, Sensitivity of the Human Eye to.** C. Trolle-Lassen. (*Pharm. Weekbl.*, 1958, **93**, 148.) Healthy individuals were used for the tests. A non-irritant solution was instilled into one eye, and the test solution into the other. The experiment was repeated with other strengths of solution and from the results the threshold of irritation was calculated. Considerable variation was observed in the sensitivity of individuals to solutions diverging from the normal osmotic pressure and hydrogen ion concentration of the lachrymal fluid, which corresponds to a freezing point depression of 0·52° and a pH of 7·4. Solutions causing irritation in 1 per cent or less of the individuals used in the tests were considered to be non-irritant. Using this criterion solutions of freezing point depression 0·41° to 0·77° (equivalent to 0·7 to 1·4 per cent of sodium chloride) and pH 7·3 to 9·7 were classified as non-irritant. Thus the eye tolerates hypertonic rather than hypotonic, and alkaline rather than acid solutions. It was observed that substances such as urea, although they pass freely through animal membranes, affect the osmotic pressure of solutions for instillation, and should be taken into account in adjusting the freezing point of solutions for use as eye-drops. G. B.

## PHARMACOGNOSY

***Salvia officinalis* L., Periodical Daily Variations in the Content of Volatile Oil in the Leaves of.** R. Schib. (*Pharm. Acta Helvet.*, 1958, **33**, 32.) This investigation shows that the volatile oil content of *Salvia officinalis* has significant and regularly repeated variations during a 24-hour period. Thus it was found that the maximum content occurred between noon and 4 p.m., and the minimum between 11 p.m. and the early hours of the morning. The difference between maximum and minimum contents, referred to the crude fibre (since this was the least variable factor) was found to be between 34 and 45 per cent of the minimum content. In fully developed leaves the curves oscillated between maxima and minima, whereas very young plants showed a small secondary maximum between 1 and 5 a.m. The curves of the diurnal variations were found to be approximately parallel with those of air temperature. Collections were made at 4-hourly intervals. In order to minimise the sources of error which occur in the investigation of daily variations of phytochemical processes, leaves of as far as possible the same age and size were gathered from the same plant, preferably from the same height of insertion. A further series of investigations was made on seedlings from uniform seed which showed the first three leaf pairs, leaves from different plants being collected. At each collection climatic conditions were noted, e.g. temperature, wind, sunshine and the official figure for the relative humidity. The leaves were immediately wrapped in gauze and dried for 2 days in a closed tin containing lime. The variations were referred to fresh weight, dry weight and to weight of fibre, and all results showed the same trend, while those based on weight of crude fibre were considered to be least affected by other variables. The oil was determined by an oxidation method having a scatter of only ±0·5 per cent. D. B. C.

## PHARMACOLOGY AND THERAPEUTICS

**Azacyclonol in the treatment of Schizophrenia.** S. Gray and A. D. Forrest. (*Brit. med. J.*, 1958, **1**, 374.) From a controlled trial of azacyclonol in 40 chronic schizophrenic patients it would appear that the drug is unlikely to prove of great value in the treatment of this type of patient. Better results, however, were obtained in 18 patients whose illness was of more recent onset; 13 of the patients improved and 6 were discharged from hospital. It is suggested that azacyclonol has a central stimulant action and part of the poor results in the controlled trial may have been due to the masking effects of excitement and over-activity which made the patients' mental disturbance more obvious during the interview. Also the dosage in the controlled trial was less, 60 mg. daily, as compared with a range of 60 to 180 mg. daily in the clinical trial. S. L. W.

**Bemegride, Further Aspects of the Analeptic Activity of.** A. Shulman and G. M. Laycock. (*Aust. J. exp. Biol. med. Sci.*, 1957, **35**, 421.) It is already known that bemegride (β-methyl-β-ethylglutarimide) is an effective antagonist to narcosis induced by a wide variety of barbiturates and by substances structurally related to the barbiturates such as 2:4-diketothiazolidine,5-phenyl-2:4-diketothiazolodine, the monoureides bromural and carbromal, and β-methyl-β-(butyl, amyl or hexyl)-glutarimide. This work is extended by determining the analeptic potency of bemegride as shown by its ability to reduce the sleeping time of mice given a prior dose of a hypnotic bearing a structural resemblance to the barbiturate ring system. It was found that bemegride significantly reduced the hypnotic activity of glutethimide, Persedon (3:3-diethyl-2:4-diketo-1:2:3:4-tetrahydropyridine), Nodular (3:3-diethyl-5-methyl-2:4-diketopiperidine) and Dolitrone (5-ethyl-6-phenyl-2:4-diketometathiazane). M. M.

***p*-Biphenylmethyl-((±)-tropyl-α-tropinium) Bromide, Pharmacology of.** L. Gyermek and K. Nádor. (*Arch. int. pharmacodyn.*, 1957, **113**, 1.) The pharmacology of a new antiacetylcholine compound, Gastropin, is described. It is found to possess moderate antiacetylcholine effects on the isolated guinea pig ileum, rat uterus and frog heart. In the anaesthetised cat or dog it causes a transient fall in blood pressure, inhibits bladder contractions, decreases gastro-intestinal motility and salivary flow, counteracts the vagal slowing of the heart and blocks the superior cervical ganglion. In guinea pigs it is a potent broncho-dilator agent. It also possesses a moderate mydriatic action and weak neuro-muscular blocking activity. The toxicity of Gastropin lies in about the same range as that of atropine methyl bromide. Thus it may be seen that this substance, as well as possessing moderate anticholinergic activity, also possesses ganglion blocking action. On the basis of these pharmacological results Gastropin is now being tested clinically. M. M.

**Chlorothiazide, Electrolyte Excretion as Influenced by.** K. H. Beyer Jr., J. E. Baer, H. F. Russo and R. Noll. (*Science*, 1958, **127**, 146.) Chlorothiazide (6-chloro-7-sulfamyl-1:2:4-benzothiadiazine-1:1-dioxide) induces changes in electrolyte excretion similar to those caused by organomercurial diuretics. Under reasonably normal conditions of acid-base balance, the effect of chlorothiazide is to increase the excretion of sodium, chloride and water. The excretion of potassium and bicarbonate is increased slightly, if at all. The effect of this drug on the ratio of sodium to chloride excreted per unit time varies

according to the state of electrolyte balance. This chloruresis distinguishes it from the carbonic anhydrase inhibitors that cause a predominant increase in bicarbonate rather than in chloride excretion. Chlorothiazide is also capable of counteracting the sodium and fluid retention induced by steroids such as 9α-fluorohydrocortisone. M. M.

**Glucose Nitrates, Vasodilator Action of.** D. O'Meara, D. M. Shepherd and G. B. West. (*Arch. int. pharmacodyn.*, 1958, **113**, 432.) Relatively little evidence is available as to the factors which may be of importance in determining the extent and duration of the vasodilator action of organic nitrates. A series of glucose derivatives, some partially nitrated, others fully nitrated, were examined to determine the relationship between their molecular structures and their pharmacological actions. Methyl β-D-glucoside was chosen as the parent compound, since its ring structure and stereochemical configuration are fixed and its nitrates are readily obtained in the pure crystalline form. The vasodilator activity was assessed by measuring the extent and duration of the fall in blood pressure of a dog after intravenous injection of 1 mg./kg. of each nitrate and comparing the response with that of erythritol tetranitrate. It was found that compounds containing one or two nitrate groups per molecule were inactive, but those containing three or four nitrate groups possessed depressor activity comparable with that of erythritol tetranitrate. In no case were the depressor effects diminished by prior administration of atropine and/or mepyramine. On oral administration to dogs, methyl β-D-glucoside tetranitrate produced a greater and more prolonged fall in blood pressure than did erythritol tetranitrate. No correlation was found between the depressor activity and the amount of nitrite ion released by the nitrates on alkaline hydrolysis. M. M.

**Methylpentynol Carbamate.** A. H. Galley and P. Trotter. (*Lancet*, 1958, **1**, 343.) Methylpentynol carbamate was given as premedication for dental operations under local anaesthesia to over 10,000 ambulant outpatients in the following dosage: Patients without visible signs of apprehension, two 100 mg. tablets; mildly apprehensive patients, four 100 mg. tablets; very apprehensive patients, 100 mg. per stone bodyweight. Of the 10,000 patients so treated 90 per cent showed no apprehension during operation, 7 per cent were apprehensive, and 3 per cent were very apprehensive. Methylpentynol carbamate took longer to act than methylpentynol, but the effect lasted longer. It was also shown to possess advantages over pentobarbitone for premedication in ambulant patients before dental extractions under general anaesthesia. The best results were obtained when it was reinforced with hyoscine sublingually in a dose of 1/150 gr. to children up to 12 years of age, and 1/75 gr. to those over this age. Used with either hyoscine or atropine, it was also found preferable to pentobarbitone as premedication before general surgical operations under general anaesthesia. After barbiturates, withdrawal or struggling is the usual reaction to an intravenous injection, but after methylpentynol carbamate this rarely occurs. Either the drug is to some extent analgesic or it provides a central nervous sedation, in contrast to the increased nervous excitability produced by the barbiturates. In contrast to the barbiturates, laryngeal spasm does not occur after giving methylpentynol carbamate. It therefore provides a much easier change-over to ether after induction with nitrous oxide and oxygen and laryngospasm is rare during throat operations where light anaesthesia is maintained and an endotracheal tube is not used. It does not cause respiratory depression, and has a low toxicity and no undesirable side-effects. S. L. W.

# BOOK REVIEW

*AN INTRODUCTION TO PHARMACOLOGY AND THERAPEUTICS*, by J. A. Gunn with the assistance of J. D. P. Graham. Ninth Edition. Pp. ix + 327 (including Index). O.U.P., London, 1958. 18s. (U.K. only).

It is a pleasure to see a new edition of a small textbook which has served many medical students well, and to find that it is keeping up to date, without losing its original flavour or growing unduly in size. The first edition was written in 1929 with the aim of achieving brevity by selection and arrangement and it is remarkable how well this aim is still achieved, in the face of a devastating increase in the amount of knowledge from which the selection is to be drawn. The new edition retains an unhurried, but not unbusinesslike style which is pleasant to read and encourages its steady perusal. It is possible to argue about the selection: it could hardly be otherwise, for what two pharmacologists would agree what to include in 300 small pages with all the subject to choose from? Here there is all factual material, and no discussion of how the facts are discovered; therapeutic trials are not mentioned in the index, the value and defects of evidence from experiments in animals are passed by, and there are no references to the original literature. Since an unpredictable amount of the factual material is quite likely to be displaced in importance quite soon by new discoveries, and since the students who read this book will be heavily bombarded with such discoveries and their counterfeits, a guide to the evidence is the most urgent need of contemporary students, and it may be regretted that there is no development of a critical apparatus in this introduction to pharmacology. The factual material itself, though not lacking in up-to-date material, retains more of the early twentieth century than might appear in a brand new book: it is surprising still to meet references to chlorosis, and questionable whether arsenic compounds deserve nearly twice as much space as barbiturates. But the simplicity of presentation has much to commend it, and the students who want the bare facts quickly (and cheaply) will be well served by this book.

MILES WEATHERALL.