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*Editor: C. H. Hampshire, C.M.G., M.B., B.S., B.Sc., F.P.S., F.R.I.C.*

*Associate Editor: G. Brownlee, D.Sc., Ph.D., F.P.S.*

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# REVIEW ARTICLE

## THE PRESENT STATUS OF THE CHEMOTHERAPEUTIC DRUGS

By S. R. M. Bushby, M.Sc. (Brist.), Ph.D. (Lond.)
*Wellcome Research Laboratories, Beckenham, Kent*

### The Sulphonamides

The sulphonamides may eventually be replaced by antibiotics with wider antibacterial activity but they continue to be very popular in general practice because of the ease with which they are administered, and their relative freedom from serious side effects. Hypersensitivity is probably their only common disadvantage especially when applied locally in skin infections.

Although experimentally the sulphonamides are the least active of the chemotherapeutic agents they are sometimes more efficient clinically than the antibiotics. For instance, in a recent comparison[1] between the sulphonamides and penicillin for the treatment of pneumonia, there was no difference amongst males in the incidence of pleural complications but in females for some unexplained reason the sulphonamides were superior to penicillin; and again in controlled trials[2] in the treatment of infantile diarrhœa, sulphadiazine has proved to be superior to chlortetracycline or chloramphenicol. Also because succinylsulphathiazole and phthalylsulphathiazole provide good cover with little risk of serious side effects they have been regarded[3] as more desirable than the wide-spectrum antibiotics for prophylactic therapy against bowel infection in abdominal surgery.

The sulphonamides are also recommended for combined therapy, e.g., with streptomycin in *H. influenzæ* meningitis[4] and in brucellosis[5], or with neomycin as an intestinal antiseptic[6].

### Penicillin

Penicillin is often reputed to be the least toxic of all the antibacterial drugs and therefore it may be surprising to read that "To-day penicillin heads the list of medicinal agents in the frequency, diversity and severity of the sensitivities which it induces. In current experience it has replaced foreign serum as the commonest cause of fatal shock. It is responsible for a growing number of deaths due to irreversible vascular allergy, e.g., periarteritis nodosa[7]." Unfortunately this statement is well supported by published examples and the seriousness of the anaphylactic reactions is emphasised by the suddenness with which death can follow an injection. One patient had been given three uneventful courses of penicillin, then a penicillin troche caused a "queer feeling" in the chest and a brief fainting spell. Three months later the patient died within seconds of being given an intramuscular injection of penicillin[7]. Similar cases of hypersensitivity have been published by other workers, and reports of reactions, happily not often fatal, appear as a slow but steady stream to support

these statements. Procaine penicillin is especially liable to cause these allergic reactions[9].

Fortunately, these severe reactions can be prevented[7,8] by enquiring before any penicillin is administered whether the patient or parents of the patient are allergic subjects, and if penicillin has been used before (it is unlikely that there will be a severe reaction on first contact with the drug), whether there were any reactions such as swelling or itching at the site of injection, rashes, wheezing or fainting. Any patient suspected of sensitisation should first be tested intradermally, the test being observed especially for a delayed reaction, but a negative reaction will not however, necessarily exclude a state of hypersensitivity. Desensitisation has been accomplished by the usual procedure of injecting daily increasing doses of the drug.

Sensitisation of workers preparing and administering chemotherapeutic drugs is a genuine hazard that needs guarding against. A 3·5 per cent. incidence of sensitisation in nursing staff, quoted in a Memorandum from the Ministry of Health[10], emphasises the need for precautions. The greatest risk, according to this report, is incurred when working with streptomycin and the risk with penicillin is about half as great. In every case the hypersensitivity took the form of a skin manifestation, though in several cases there was also angioneurotic œdema. The sensitisation may develop after the first contact with the drug or after as long as 5 years continuous contact. Preventive measures consist of avoiding direct contact, but if contact accidentally occurs the antibiotic should be removed by washing with copious running water. Desensitisation is difficult but feasible[11].

Penicillin still remains the most active of the antibiotic drugs on a weight basis but its rate of clearance from the body has necessitated large and frequent doses. The use of depot preparations has undoubtedly helped to overcome this disadvantage, but it is only the oily ones, especially those of procaine penicillin, which are really efficient depots giving consistently detectable blood levels for 24 hours or longer. But even these are probably unsatisfactory for the treatment of deep and walled-off foci of infection especially when the organism is relatively insensitive[12].

A new repository penicillin, *NN'*-dibenzylethylenediamine dipenicillin G, benzathine, has recently been introduced[13]. It is extremely insoluble in water (about 0·02 per cent.) and when injected a dose of 300,000 units gives detectable blood levels over periods of up to 17 days[14]. But these levels are intermittent and low intermittent levels are said to present the optimum conditions for the emergence of penicillin-resistant organisms[15]. Nevertheless these infrequent injections apparently prevent the recurrence of rheumatic fever by eliminating the streptococcal-carrier state, and single injections of 600,000 or 1,200,000 units were effective in the treatment of 36 of 49 adults with lobar pneumonia[16]. This salt is also available as a mixture with procaine penicillin, the object being to ensure higher initial blood levels.

The soluble penicillin salts are poorly absorbed after oral administration and this route has therefore not been recommended except in

the treatment of infants infected with sensitive organisms, such as the pneumococcus[17], or of adults infected with the gonococcus. The addition of an antacid to the penicillin is claimed to protect it from the acid of the stomach and thus to give more consistent blood concentrations. Daily doses of 48,000 I.U./lb. of body weight taken in divided doses without an antacid have, however, been shown[18] to give reliable bacteriostatic blood concentrations in infants, provided the drug was taken on an empty stomach; an antacid did, however, halve the necessary dose.

A recent innovation for oral administration is the insoluble salt benzathine, mentioned above. Claims have been made that it produces more consistent, though not necessarily higher, blood levels than comparable doses of the soluble salts[19], but this has not been substantiated by other workers[20,21], who find it behaves no differently from the soluble salts. That it should behave similarly to the soluble salts is not surprising, for it rapidly hydrolyses at the *p*H of normal gastric juice and significant hydrolysis also occurs in slightly alkaline solutions[22]. It would seem therefore that the only real advantage of this new salt is that it is stable as an aqueous suspension and can be used as an elixir in a palatable form for children.

Until recently only inorganic salts of benzylpenicillin have been used in clinical practice but the diethylaminoethyl ester of benzylpenicillin has been introduced on the grounds that it possesses an affinity for lung tissue and is excreted in relatively large amounts in the sputum[23]; animal experiments suggest that this affinity is more marked when the lungs are infected with *H. pertussis*[24]. Also, high concentrations of penicillin are present in the cerebrospinal fluid after intramuscular injections of this ester[25]. But in spite of the claims made for it, conclusive clinical proof showing any evidence of superiority over ordinary penicillin is still lacking[12]. This penicillin represents a derivative of a new type for the esterification is on the carboxyl carbon of the penicillin molecule, but in 0·9 per cent. sodium chloride solution at *p*H 7·3 and 37° C. it undergoes 50 per cent. hydrolysis within 23 minutes, and although its toxicity is low when given orally or intramuscularly it is highly toxic when given intravenously[26].

Although penicillin has now been widely used for seven or eight years, resistant organisms have not become a serious problem, except with staphylococci[27], and fortunately, alternative drugs are usually available against the resistant strains. The incidence of penicillin-resistant staphylococci naturally tends to be high in hospitals where the use of penicillin eliminates the sensitive ones and where wound infections arise mainly from carriers and cross-infection. In one survey[28] of 915 strains isolated from in-patients, 65 per cent. were resistant to penicillin, 28 per cent. were resistant to streptomycin, 8 per cent. to chlortetracycline and oxytetracycline, and only 1 per cent. to chloramphenicol. Of the strains resistant to chlortetracycline and oxytetracycline 98 per cent. were resistant to penicillin. The incidence of resistant strains in the staff of this hospital is of ætiological significance; 54 per cent. were found to be nasal carriers of staphylococci and of these 47 per cent. were carrying resistant strains. Similar findings have been reported by other workers[29].

Penicillin-resistant strains are usually sensitive to chloramphenicol and often to the newer antibiotics, erythromycin and carbomycin. Bacitracin is also active against most of these resistant strains but unfortunately this antibiotic is not suitable for parenteral use.

There is experimental evidence that the exposure of an organism to the action of one antibiotic changes its resistance to another[30], and in America the incidence of penicillin-resistant strains of staphylococci is reported[27] to have fallen during the last two years, and the fall was attributed to the widespread use of bacitracin and the tetracycline antibiotics.

## Streptomycin

This antibiotic is still the most important drug in the treatment of tuberculosis (*vide infra*). It is unfortunate that organisms so readily develop resistance to it because it is also active against many Gram-positive and Gram-negative organisms, and when used for short periods causes few side reactions. In spite, however, of this weakness it has proved very effective in some infections. *In vitro* against *Shigella sonnei* it is more effective than chloramphenicol or the tetracyclines, and in 16 acute cases of dysentery oral streptomycin produced clinical cures within 24 hours and bacteriological cures with no relapses within 6 days[31]. It is still probably the most active antibiotic available for *Proteus vulgaris* and *Klebsiella pneumoniæ* infections[12].

In order to reduce the rate at which organisms develop resistance to streptomycin, this antibiotic is now frequently used in combination with other drugs[12]; combined with sulphadiazine it is usually effective in *H. influenzæ* meningitis[4] and combined with oxytetracycline it is strongly recommended for the treatment of brucellosis[5].

## Chloramphenicol

This antibiotic is still unique in being the only one that is manufactured synthetically more cheaply than it is produced naturally.

Although chloramphenicol is one of the wide-spectrum antibiotics, i.e., is bacteriostatic against many Gram-positive and Gram-negative organisms, rickettsia and the larger viruses, it is most effective when used against Gram-negative bacilli[12]. The tetracyclines and the polymyxins have high *in vitro* activity against *Salmonella typhosa*, but chloramphenicol is the only drug which is of real value for the treatment of typhoid fever[12,32]. Again, in a controlled trial with chlortetracycline, chloramphenicol and sulphadiazine in the treatment of infantile diarrhœa, which recent investigations suggest is due to certain strains of *Bact. coli*, chloramphenicol proved superior to chlortetracycline although it was inferior to sulphadiazine. In the treatment of urinary infections by *Proteus vulgaris*, it is frequently effective because many strains of this organism are sensitive to it[33]. In staphylococcal infections it is usually not as effective as penicillin or the tetracyclines but as many strains are now resistant to these other antibiotics, chloramphenicol is becoming increasingly more valuable against staphylococci.

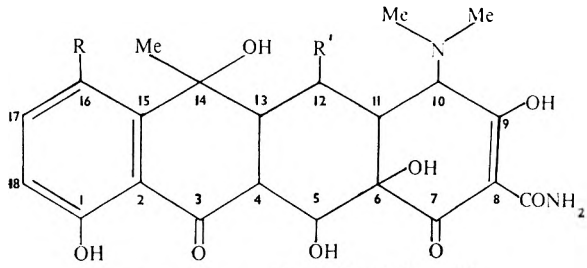
Chloramphenicol is also particularly valuable in the treatment of pulmonary infections not only because of its high activity against most of the bacteria found in the respiratory tract but also because of its activity against the virus of viral pneumonia. In an extensive trial[34] it was also shown to be of value in the treatment of whooping cough, but it had to be used early in the disease and even then the effects were not dramatic. Chlortetracycline was also used in the same trial and it produced similar results.

Although chloramphenicol is the most effective drug available for the treatment of typhoid fever it is far from ideal; the relapse rate is high, and complications such as perforation and cholecystitis are not infrequent[12]. These relapses may be due to inadequate treatment but most probably to the drug being only bacteriostatic.

Large quantities of chloramphenicol were used in America before it was realised that the drug occasionally produces aplasia of one or more elements of the hæmopoietic system[35], and recently the Council of Pharmacy and Chemistry has considered it necessary to advise that the use of chloramphenicol be restricted to the treatment of typhoid fever and other serious infectious diseases caused by organisms resistant to other chemotherapeutic agents. Investigation[36] of 31 cases of aplastic anæmia associated with the use of chloramphenicol in this country has shown that in adults the total dose of the drug should not exceed 26 g. and the length of treatment should not be longer than 10 days. In children the maximum total dose should not exceed 100 mg./kg. over a period not longer than 7 days.

## The Tetracycline Antibiotics

The very similar antibacterial activity, and "cross-resistance"[37], of "aureomycin" and "terramycin" strongly suggested that the two antibiotics were related in spite of their being produced by different species of *Streptomyces*. The closeness of the relationship was, however, not fully realised till the chemists of the two companies responsible for their respective developments gave details of the structural formulæ of the antibiotics[38]. Among antibiotics this chemical configuration is unique and they differ only in that "aureomycin" has a chlorine atom at carbon 16, and "terramycin" has a hydroxyl atom at carbon 12.




Aureomycin R = Cl; R′ = H

Terramycin R = H; R′ = OH

The terms chlortetracycline and oxytetracycline have now been accepted as the descriptive names for "aureomycin" and "terramycin" respectively.

These antibiotics have wide antibacterial and antiviral activities similar to those of chloramphenicol but in contrast to chloramphenicol they are particularly effective against the Gram-positive cocci. They have proved to be of especial value against penicillin-resistant staphylococci, but recently a high proportion of these penicillin-resistant strains have proved to be resistant to them as well[12,28]. Like chloramphenicol, they are bacteriostatic only and the carrier rate after therapy is much higher than after using a bactericidal drug, e.g., penicillin[39].

Being absorbed like chloramphenicol, from the gut, these antibiotics are usually taken orally, and they are effective in the treatment of a variety of infections, especially of pneumonia, urinary infections[40], actinomycosis[41], brucellosis[43], and non-specific urethritis[44]. Side reactions are rarely serious, and consist of nausea, vomiting and loose stools. Intravenous preparations are available but are seldom used unless the patient cannot take the drug orally; intramuscular injection of the hydrochloride has been recommended for oxytetracycline[42].

The wide antibacterial activity causes almost complete sterility of the gut and therefore interferes with the bacterial synthesis of members of the vitamin B complex, but the deficiency becomes important only after prolonged treatment. More serious however, is the growth of monilia which can sometimes invade the tissues and produce a fatal infection[45]. The risk of moniliasis, especially the pulmonary form, is such that the American Council of Pharmacy and Chemistry in 1951 issued a warning statement to be added to the labelling of this drug drawing attention to the risk. This increase in the incidence of monilia in the sputum, throat and rectum after treatment with oxytetracycline or sulphadiazine was determined in 174 patients with pneumonia[46]. Treatment with the antibiotic increased the incidence of monilia in the sputum from 32 per cent. before treatment to 61 per cent. after treatment; in the throat it was increased from 16 to 42 per cent. and in the rectum from 0 to 59 per cent. Treatment with the sulphonamides caused no immediate increase, but 2 to 4 days later there was an increase, due perhaps, according to the investigator, to cross-infection from patients treated with the antibiotics. The pruritis and rectal soreness which so frequently accompanies the oral administration of the wide-spectrum antibiotics has also been attributed to the moniliasis[47].

The cause of the sudden growth of monilia during treatment with these antibiotics is still unknown but it has been attributed either to the elimination of the sensitive organisms thus permitting a vast increase in the few resistant monilia normally present, or to changes in the mucous membrane, which then permits invasion by organisms normally unable to penetrate the healthy mucosa, or to a direct stimulation of the growth or virulence of monilia. It has been shown[48] that *Candida albicans* and chlortetracycline are non-toxic when injected singly intraperitoneally into mice but together they are fatal, due, it is suggested to chlortetracycline lowering the animal's resistance. In other experiments[49]

chlortetracycline has stimulated the growth of *Candida albicans*, but the fact that chloramphenicol, streptomycin and oxytetracycline, especially the latter, failed to produce the same stimulating effect makes unlikely the suggestion that stimulation is the cause, since moniliasis has followed treatment with all these drugs.

Attention has recently been drawn[50] to another serious complication, antibiotic enterocolitis, following the oral use of the wide-spectrum antibiotics. The direct cause of this infection is not always known but sometimes it is due to the replacement of the normal intestinal flora by strains of organisms not susceptible to the antibiotic and the result can be fatal, especially when the organism is *Staph. aureus*. These complications are, however, not contra-indications for the use of these very powerful antibiotics but stress the need for restraint in using them as prophylactics.

Achromycin or tetracyn is a more recently introduced tetracycline antibiotic which differs chemically from the earlier two members of the series by the absence of both the chlorine and the hydroxyl groups; it has been given the descriptive name, tetracycline. It has been prepared from both chlortetracycline and oxytetracycline and is also produced naturally by a species of *Streptomyces* isolated from Texas soil[51]. Its antibacterial activity is very similar to those of chlortetracycline and oxytetracycline but it is more stable in solution than is chlortetracycline and it does not stimulate the growth of *Candida albicans*. Resistance is claimed to develop more slowly and it is said to be less toxic, and cause less gastro-intestinal disturbance[12,52]. Used in the treatment of 179 patients[53] mainly with urinary and respiratory infections it had effects similar to those that would have been expected from treatment with the other two tetracyclines, except that the side-effects were probably less.

### Erythromycin and Carbomycin

These new antibiotics are derived from two species of *Streptomyces* and are most active against Gram-positive organisms. They are readily absorbed from the gastro-intestinal tract and having similar activity to penicillin the main interest in them is for use against strains of staphylococci resistant to penicillin and the tetracycline antibiotics.

Erythromycin is a basic substance, and it is active only against multiplying organisms. It is effective in staphylococcal infections, but unfortunately resistance quickly develops[12]. *In vitro*, according to one report[54], after 3 to 5 subcultures staphylococci sensitive to 0·4$\mu$g./ml. became resistant to 100$\mu$g./ml., and according to another[55], within one month of adopting erythromycin for general use in a hospital, strains of resistant staphylococci were isolated from the nose and throat of the staff and within 5 months the carrier-rate of these resistant strains reached 75 per cent. There is however no cross resistance with any of the other antibiotics in common use, except with carbomycin[56]. It is also effective in streptococcal infections; in the treatment of scarlet fever and in the prevention of the suppurative complications it is apparently as good as penicillin[57].

Carbomycin, a crystalline monobasic substance, is also highly active

against staphylococci and even more so against pneumococci and hæmolytic streptococci. After oral administration the blood concentration is low but the tissue concentration is reported to be high[58]. There are as yet few reports of its clinical use and those available are not promising; pneumococcal pneumonia appears to respond less dramatically than with the more usual drugs[12], and the response in staphylococcal infections is often even more disappointing[12]. It appears, however, to be of some value in the treatment of urinary infections, especially when the causal organism is the enterococcus[59]. Resistance to carbomycin readily develops *in vitro* and there is cross-resistance with erythromycin[60].

Both erythromycin and carbomycin are active against rickettsia and some of the larger viruses and, although there is yet no evidence of rickettsia developing resistance to antibiotics, they may be useful as alternatives to the tetracyclines and chloramphenicol for the treatment of typhus, etc.

## The Polypeptide Antibiotics

There are several antibiotics of this class but only bacitracin and the polymyxins are used in man.

Bacitracin is active against many Gram-positive organisms but because of renal toxicity[61] it is usually only used topically, and when applied to the surface of the brain it is apparently far less toxic than penicillin[12,27].

The polymyxins are a group of five antibiotics produced by different species of *B. polymyxa*. Three of them, polymyxins, A, C and D cause transitory proteinuria but polymyxins B and E are almost, if not entirely, free from this effect[62,63]. It is unfortunate that the first polymyxins to be used were the renal toxic polymyxins A and D, for they have undoubtedly helped to create the impression that all the polymyxins are unfit for parenteral use. The adoption of the same name for each of them has also contributed to this impression, for frequently the suffix is omitted when reference is made to any of them. Both polymyxins B and E cause mild paræsthesia when used parenterally, and also some pain at the site of injection but this is less with polymyxin E than with polymyxin B[62,63].

The polymyxins are active against almost all bacteria but they are at least a 100 times more active against the Gram-negative bacilli than against other organisms. As they are not absorbed from the gut, they must be given parenterally for systemic use[62], but they are rapidly bactericidal and injected intrathecally rapidly sterilise the cerebrospinal fluid of patients with meningitis due to sensitive organisms, e.g., *H. influenzæ*[64]. Experimentally the polymyxins are very efficient against *Salm. typhosa*[62], but the produce little or no effect when used in the treatment of typhoid fever[65,66]. In practice they have proved to be the best available drugs for the treatment of *Ps. pyocyanea*[67] infections especially in meningitis[68], burns[69] and eye infections[70], and they are also very effective in selected cases of urinary infections.

The polymyxins and bacitracin have bactericidal activities which are complementary and as the drugs rarely cause hypersensitivity when used

topically[12], they form an excellent combination for the local treatment of infected wounds and dermatological lesions[72], conditions in which most other chemotherapeutic drugs have limited uses because of hypersensitivity. This combination is also recommended[12,27] for reducing the bacterial flora of the intestine since neither of the drugs is absorbed from this site.

## Neomycin

Neomycin is particularly active against the tubercle bacillus[73], but it is too toxic for systemic use[74]. It is however, bactericidal for many other bacteria and as it is not absorbed from the gastro-intestinal tract it is used as an intestinal antiseptic, mainly in combination with bacitracin[27], the tetracylines[75], and phthalylsulphathiazole[6]. It is also poorly absorbed from wound surfaces and applied topically for the control of surgical infection it has been particularly useful against *Proteus vulgaris*[27].

Although organisms do not readily develop resistance to neomycin, those that do show cross-resistance with streptomycin[76]. Neomycin as a 0·5 per cent. cream was successfully used alone for the treatment of 93 patients with pyogenic skin infections due to staphylococci resistant to penicillin, chloramphenicol and aureomycin; none developed hypersensitivity[77].

## The Antitubercular Drugs

Much progress has been made in the treatment of pulmonary tuberculosis by the use of chemotherapeutic drugs and it seems unlikely that any new drug will be discovered that is more active than some of the present ones. The value of the present ones is probably limited only by the ease with which the tubercle bacillus develops resistance to them and by the inaccessibility of the organisms in the more chronic forms of the disease. The development of resistant organisms has, however, been much reduced by the concomitant use of two or more drugs and advances in surgery are helping to overcome the limiting effect of the disease by enabling the irreparable portions of the lungs to be removed. The surgical advances are, however, only possible because the drugs are able to control the acute spread of the disease which almost invariably follows surgical interference.

The sulphones were the first drugs to show definite though slight *in vitro* and *in vivo* antitubercular activity[78,79], and clinically they were of some value in the treatment of pulmonary tuberculosis[80,81], but in 1946, even before the trials were completed, streptomycin was showing in animal experiments[82,83] how vulnerable is the tubercle bacillus to a really efficient drug. Before long there were very favourable reports of the use of streptomycin in patients with miliary and meningeal tuberculosis[84,85], the two conditions in which previously the prognosis had been hopeless.

Two drawbacks to streptomycin soon became apparent. Firstly, the initial clinical improvement was often not maintained, especially in the

more chronic forms of the disease, due to the organisms becoming resistant to streptomycin[86]. Secondly, streptomycin damaged the nucleus of the eighth nerve causing vertigo and ataxia from vestibular disturbances, and hearing impairment, often to the extent of complete deafness, was caused by damage to the auditory mechanism[87]. This toxicity has now been minimised by reducing the dose of streptomycin[12], and giving it less frequently and for shorter periods. In 1948 dihydrostreptomycin was introduced[88], on the grounds that it was less toxic than the parent antibiotic, but experience has shown that it causes greater hearing impairment, and this is more serious than vestibular damage because the latter can be recognised early when the damage is reversible whereas the auditory nerve damage is insidious and not reversible[12,74,89]. However, from the results of a questionnaire[90] sent to 19 sanatoria in this country it appears that the risk of complete deafness from dihydrostreptomycin is small provided the daily dose does not exceed 1 g. Since the two streptomycins have additive actions on the tubercle bacillus and yet tend to produce different side effects, streptomycin and dihydrostreptomycin are being given in combination in an attempt to lessen the side effects[89].

The importance of streptomycin-resistant organisms in the sputum of any particular patient is difficult to assess[91]. In one series of cases[91], although resistant organisms were isolated from the 42nd day of treatment onwards, and the number of bacilli present in the sputum having fallen initially rose again, the early clinical improvement was maintained in most of the patients, in spite of the persistence of resistant strains. Moderate drug resistance seems to be of little clinical significance[92].

In 1946, *p*-aminosalicylic acid was shown to have high *in vitro* antitubercular activity[93] and to be active in guinea-pigs[04], and early clinical reports suggested that it was also active in the treatment of tuberculosis in man[95]. Although 1 to 10 $\mu$g./ml. inhibit growth of the tubercle bacillus *in vitro* it is less active in animal experiments than streptomycin[96] and more recent clinical reports showed that although it may have some effect it is inferior to streptomycin. Tubercle bacilli develop resistance to *p*-aminosalicylic acid but to a lesser degree than to streptomycin[74,86,92,97]. It has low toxicity, usually causing only anorexia, mild nausea, and diarrhœa but it not infrequently causes hypersensitivity[97]. The sodium salt causes less gastro-intestinal upset than the free acid but, due to the rapid rate of excretion, the dose of *p*-aminosalicylic acid has to be large, 12 to 15 g. per day, and the quantity of sodium may be too high for patients requiring a low sodium intake[89]. The calcium salt is reported to cause even less gastro-intestinal upset than the sodium salt[98].

By now the use of *p*-aminosalicylic acid would probably have been limited to chronic cases requiring prolonged treatment but for the fact that given with streptomycin it delays the development of streptomycin-resistant strains. This effect has been demonstrated experimentally, both *in vitro* and in animals[99,100], and clinically in man[86,92,97]. In a trial in this country[86], patients treated with combined *p*-aminosalicylic acid (20 g. daily) and streptomycin (1 g. daily) showed improvement somewhat greater

than those who received streptomycin alone, and while from the 49 patients treated with streptomycin alone, 33 streptomycin-resistant strains were isolated, only 5 such strains were recovered from the 48 patients on combined therapy.

In 1946 a new class of compounds, the thiosemicarbazones, was shown[101] to be effective *in vitro* and experimentally *in vivo* against the tubercle bacillus, and in Germany, *p*-aminobenzaldehyde thiosemicarbazone (T.B. 1/698) has been widely used in the treatment of tuberculosis[102,103]. In this country and in America, however, it has been little used because of toxic side effects, which have included malaise, dizziness, photophobia, liver necrosis and bone marrow hypoplasia[102], This drug has no effect on miliary or meningeal tuberculosis[102], it is probably less active than *p*-aminosalicylic acid and resistant strains can be isolated from the patients as early as 4 weeks after commencement of treatment[104]. Other thiosemicarbazones have been tried, e.g., *p-iso*butoxybenzaldehyde thiosemicarbazone but the clinical effects have not differed from that obtained with *p*-aminobenzaldehyde thiosemicarbazone[105].

In 1952 the high antitubercular activity of isoniazid, *iso*nicotinyl hydrazide, was announced independently by 3 groups of workers, 2 in America[106,107], and the third in Germany[108]. Isoniazid is highly specific against mycobacteria, but it has been reported to be effective also in the treatment of actinomycosis[109]. Although the *in vitro* activity is of the same order as that of streptomycin, in animal experiments it is even more effective[106]. The first reports of its clinical use were most encouraging[110], but more prolonged use showed that the effects were only temporary[111,112], the relapses coinciding with the isolation of isoniazid-resistant strains; in one trial[112] in which 264 patients were treated, 30 still had tubercle bacilli in their sputum after 6 months treatment and of these 28 had isoniazid-resistant strains.

The importance of isoniazid resistance is even more difficult to assess than streptomycin resistance because the degree of resistance varies so much and many of the more highly resistant strains have been shown to be of very low virulence to mice and guinea-pigs[113,114,115,116], and there is the rather disturbing experimental evidence which suggests that some of the less highly resistant strains are actually made more virulent by the presence of isoniazid[115,116]. Clinical experience, however, suggests that the patients with organisms resistant to 0·2 $\mu$g./ml. but not to 1 $\mu$g./ml will continue to respond favourably to treatment with isoniazid[117].

There is no cross-resistance between strains resistant to isoniazid, *p*-aminosalicylic acid or streptomycin, and there is now much evidence to show that combined treatment with isoniazid and *p*-aminosalicylic acid or streptomycin delays the development of resistance, thus enabling the initial clinical improvement to continue. In one trial[118] doses of 100 mg. of isoniazid daily with 1 g. of streptomycin daily or 5 g. of *p*-aminosalicylic acid 4 times a day reduced the incidence of resistant strains to a degree comparable to that produced by the combination of streptomycin and *p*-aminosalicylic acid on streptomycin-resistant strains. The clinical improvements in both of these groups were satisfactory, and did not

significantly differ from each other. In a supplementary comparison in this trial the streptomycin was reduced from daily to twice weekly doses but the incidence of isoniazid-resistant strains was higher than in the group receiving the daily doses.

It is now generally agreed that isoniazid should never be used alone[74], and from the activity shown in animal experiments it was expected that isoniazid would be at least as effective as streptomycin if the development of resistant strains were prevented. In the acute human infections where the lesions are more vascular and the drug can readily reach the organisms, e.g., in the miliary and meningeal forms of the disease[119,120], this expectation appears to have been fulfilled for it appears to be even more effective than streptomycin. Although both isoniazid and streptomycin are bactericidal[121,122], histological examination of tissue from patients with miliary and meningeal tuberculosis, dying during treatment with these drugs also suggests that isoniazid is the more efficient drug because with it the lesions show resolution whereas they become fibrosed during treatment with streptomycin[123]; when combined treatment with these drugs is used the isoniazid effect predominates. The higher efficiency of isoniazid may be due to the fact that tuberculosis is essentially an intracellular infection and isoniazid penetrates cells more readily than does streptomycin[124,126]. Studies with radio-active isoniazid show that it also diffuses into caseous lesions[125].

In spite of the experimental evidence of isoniazid possessing advantages over streptomycin and that *in vitro* the bactericidal activity of the drugs together is greater than when acting alone, especially when tested at maximal therapeutic concentrations, there is no conclusive clinical evidence that the combination of streptomycin-isoniazid is more effective than the combinations, isoniazid-*p*-aminosalicylic acid and streptomycin-*p*-aminosalicylic acid in pulmonary tuberculosis[127,128]. The triple combination, streptomycin-isoniazid *p*-aminosalicylic acid, is being tried and if it proves to suppress, completely or almost completely, the development of resistant strains, then this will obviously be the combination of choice. According to one report[128], however, the combination shows no significant advantage and if this is confirmed then it would seem unwise to use streptomycin and isoniazid together. Undoubtedly these two drugs are the only really effective antitubercular drugs available and it would therefore seem better to use only the combinations of streptomycin-*p*-aminosalicylic acid or isoniazid-*p*-aminosalicylic acid so that should resistance develop, which it invariably does in a proportion of patients, there will still be available the alternative powerful drug[74].

Of the double combinations, isoniazid-*p*-aminosalicylic acid seems to be the more desirable for routine treatment as it avoids the use of the hypodermic syringe and although isoniazid is not completely free from side reactions, these are not as serious as the damage caused by streptomycin to the eighth nerve. The side reactions attributed to isoniazid have included central nervous system stimulation (hyperactive deep reflexes, twitching, insomnia, changes of temperament) and muscle weakness but no serious effects on the liver, kidney or bone marrow have been reported[89].

Many derivatives of isoniazid have been examined *in vitro*[106,129], and some have been used clinically but none has so far proved superior to the parent substance[74,110]. An analogue of nicotinic acid, pyrazinamide, also has *in vivo* antitubercular activity[130] and has been used clinically[131] but drug resistance quickly develops. Recently it has been used with isoniazid, and according to one report[132] the combination rapidly eliminated tubercle bacilli from the spleen of mice infected experimentally and in 90 per cent. of 61 patients with pulmonary tuberculosis, completing a course of 3 months duration, the sputum became negative and in 70 per cent. there were substantial radiographic improvements. The authors were of the opinion that the antitubercular activity of the combination is superior to that of any other combination in current use, but as the incidence of hepatitis was high, 6 patients developing liver damage from which one died, it is unlikely that the combination will be widely used. Similar results but with less evidence of liver damage have been reported by another group of workers[133].

Most of the controlled studies with the antitubercular drugs, singly or in a combination have been on patients with the pulmonary form of the disease but there is no doubt that they are of equal value in the treatment of extra-pulmonary lesions, e.g., meningeal or renal tuberculosis[74,134]. The treatment of tuberculous meningitis is one of the outstanding successes of chemotherapy for previously this condition was almost invariably fatal. Treatment with intrathecal streptomycin alone increased the survival rate by about 50 per cent.[74] and on a regime of intramuscular streptomycin and oral *p*-aminosalicylic acid[135] or oral and intrathecal isoniazid it is much higher[136,137]; in one report, only 6 deaths occurred in 100 cases, and in another only 1 out of 30. The administration of intrathecal drugs is however a very disturbing procedure for the patient, but as adequate concentrations of isoniazid have been shown to be present in the cerebrospinal fluid in tuberculous meningitis after oral administration[120,138], and as treatment with intramuscular streptomycin and oral isoniazid with or without *p*-aminosalicylic acid has been successful[139] the intrathecal route is probably unnecessary with isoniazid. Intrathecal streptomycin is also probably unnecessary for, although streptomycin does no pass the normal blood-brain barrier, it apparently gets through when there is a meningitis for in one series of patients 8 of 19 were alive 3 to 5 months after beginning treatment with streptomycin and all except one had received the drug intramuscularly only[140].

The tetracycline antibiotics have some *in vitro* and experimental *in vivo* antitubercular activity[141], and have been tried in pulmonary tuberculosis. Although treatment with chlortetracycline or oxytetracycline is ineffective[142], the latter apparently delays the development of streptomycin resistance, for of 66 patients given 5 g. of oxytetracycline daily in combination with 2 g. of streptomycin every third day for 120 days, at the end of treatment 25 still had tubercle bacilli in their sputa but all the strains were still streptomycin- and oxytetracycline-sensitive[143]. This effect has been confirmed by other workers[144] and it has also been shown[145] that 2 g. doses of oxytetracycline are insufficient.

Neomycin is more active *in vitro* than is streptomycin[73] against the tubercle bacillus but as it causes auditory and renal damage[12], it is not used systematically in tuberculosis[74].

Viomycin is active *in vitro* against some Gram-negative organisms, but it is more active against the tubercle bacillus, 2·5 to 12·5 $\mu$g./ml. inhibiting most strains[146]. In animals, however, it is less effective than streptomycin, having activity approximately equal to that of *p*-aminosalicylic acid[147]. It was introduced in 1951[148], but it has been little used in man because toxic side reactions are common. The most important of these have been renal damage, vestibular disfunction, partial deafness, and pain at the site of injection[149], and they have occurred even when the patients were given only 2 g. every third day. In future it will probably be used in conjunction with other drugs only in cases where the organisms haue become resistant to the less toxic drugs. Trials of such combinations are being run, using *p*-aminosalicylic acid and streptomycin, but the preliminary results are not promising for the incidence of toxic reactions and of the emergence of viomycin-resistance strains is high[150].

## Antileprotic Drugs

Leprosy is caused by *Myco. leprœ*, and although this organism has much in common with *Myco. tuberculosis*, it has never been cultivated with certainty, or produced a progressive disease when inoculated into animals, thus making the usual antibacterial screening tests impossible for detecting the antileprotic activity of drugs. The only method available has been to try drugs in persons suffering from leprosy but even this means of assessment is extremely difficult for the disease runs a very chronic course during which it shows a natural tendency to progress and retrogress.

When the first antitubercular compounds, the sulphones, were shown to have some effect against tuberculosis it was natural to try them in the treatment of leprosy. The first sulphones used were the di-substituted forms of 4:4′-diaminodiphenylsulphone (dapsone), e.g., promin, diasone and sulphetrone, and these were reported to be very active in the lepromatous form of the disease.

The di-substituted sulphones were chosen because of the high toxicity of the parent substance, but small doses of dapsone (400 mg. twice weekly) have more recently been shown to be well tolerated and as effective as the larger doses of the less acutely toxic but more expensive derivatives There is some evidence that the derivatives owe their activity when taken orally to being broken down to dapsone[151], but the di-substituted sulphone, sulphetrone, is also active when given parenterally[152] when little or no breakdown occurs. Sulphetrone, however, contains an appreciable quantity of mono-substituted dapsone (semi-sulphetrone)[153] and it is possible that sulphetrone owes its activity to this, for at least one other mono-substituted form of dapsone is active; i.e., the monoacetyl ester of dapsone, sulphone cilag[154].

The activity of these sulphones with only one free amino group is interesting in view of the fact that dapsone is excreted almost entirely in a conjugated form, probably as a mono-substituted derivative[153]. The

antileprotic activity of dapsone may be due to this derivative and not to unchanged dapsone because the parent substance circulates in the body for only a very short time after oral administration.

All the active antitubercular drugs have been tried in leprosy. Streptomycin apparently has some activity[155], but it is not so spectacular in this disease as in tuberculosis, and it is usually regarded as less active than the sulphones. Thiosemicarbazone has definite activity[156], and isoniazid is still undergoing trial but the results so far are not very promising[157].

Whether the development of resistant organisms is the reason for the poor results from streptomycin and isoniazid in this disease is problematical, but the conditions of treatment, being so prolonged are undoubtedly conducive to the development of resistance. Resistance to isoniazid can develop very readily with *Myco. lepræmurium*[158], the organism morphologically indistinguishable from *Myco. lepræ*, and which produces a disease in rodents very similar to the human disease. The answer should come from trials now being made with combinations of the antitubercular drugs, but it may be that leprosy is a disease in which chemotherapy, especially with a bactericidal drug, cannot produce spectacular results. Patients with the lepromatous form of the disease have myriads of organisms present in the infected areas and experience with the lepromin test suggests that even if they were all killed they would continue to behave as living organisms for many weeks. In the lepromin reaction boiled bacilli are injected intradermally and these organisms may continue to produce a local reaction for many weeks. With the bacteriostatic drugs, the sulphones, morphological changes occur in the organism and eventually they appear to disintegrate into acid-fast dust. These changes may be an active process by the organisms to an unfavourable environment, and in the opinion of one authority[154] the granules are capable of reverting back to the active bacillary form if sulphone therapy is discontinued. This authority suggests that 10 to 15 years may be needed to be certain that a patient is cured of leprosy by sulphone therapy. If the morphological changes induced by a bacteriostatic drug result in a clinically inactive form then a better immediate response may be produced by bacteriostatic than by bactericidal drugs.

### Combined Therapy

Combining two or more drugs in the treatment of infection is becoming common. Combined therapy can justifiably be used for (1) delaying the development of resistant strains, (2) increasing the activity by the additive or synergistic effect of two drugs against an organism not sufficiently sensitive to the drugs when acting singly, (3) the treatment of mixed infections by drugs with narrow antibacterial spectra, (4) the treatment of infections where the causal organisms are unknown.

Precisely just how the presence of a second therapeutic drug prevents an organism developing a resistance to the first is unknown but it is generally presumed, that an organism becomes insensitive by using a metabolic pathway other than that interfered with by the drug. When

two drugs are presented with different modes of action the organism has difficulty in developing simultaneously two alternative routes, and when three drugs are present the difficulty must be immense. The use of combined therapy has found its widest application with the antitubercular drugs.

Much attention has been given to the possibility of drugs interfering with each other, and, experimentally, combination of drugs undoubtedly show enhancing or antagonistic effects. When two drugs show enhancement and the increased effect is equal to the sum of the respective activities of the two drugs, the effect is usually regarded as additive but when it is greater it is called synergism. A rather different and narrower conception of synergism has been suggested for chemotherapeutic drugs. It is based on the assumption that the more rapidly a drug kills the organism the higher is its activity and on the fact that frequently when a bacteriostatic drug is used with a bactericidal drug the former may delay or even prevent the killing of an organism by the bactericidal drug. Synergism, according to his proposed conception occurs when the early bactericidal action is increased and not when there is merely an increase in the bacteriostatic effect[1 9].

The knowledge that antagonism can occur between two drugs prevents the general use of combined therapy. According to some authorities[159,160], it is impossible to lay down rules that a certain combination of drugs will always show synergism or antagonism against a particular species or organism, because strain variations occur. Nevertheless they suggest a scheme that should be of clinical value for selecting combinations of drugs. In this scheme the drugs are divided into two groups:

(1) those that are essentially bactericidal—penicillin, streptomycin, bacitracin and neomycin;

(2) those that are essentially bacteriostatic—the tetracyclines, and chloramphenicol.

Members of group 1 are frequently synergistic with each other, occasionally indifferent but never antagonistic. Members of group 2 are only additive with each other, and members of group 2 are usually antagonistic to those of group 1

The results of these various interactions are based on bacteriological studies involving viable count on organisms exposed to different concentrations of the drugs. Such procedures are unsuitable for routine use for selecting the proper combination of drugs but a recently described technique[161] enables synergism according to the increased bactericidal conception to be deduced with the minimum of labour.

By this technique the drugs of group 1 and group 2 are antagonistic during early incubation but the effects may change on further incubation. Thus penicillin and chlortetracycline may remain antagonistic after 18 to 24 hours incubation or they may become indifferent, and streptomycin and chloramphenicol may become synergistic in 18 to 24 hours. Also the effects observed at 24 hours with any pair of antibiotics is constant for any species suggesting that it is possible to recommend suitable standard combinations of drugs.

Almost all the studies on drug interference have been made *in vitro* and some doubt may be felt as to the extent to which interference acts *in vivo* where the concentrations of the drugs vary and the host's natural defences must play an important part in the effectiveness of treatment. Experiments in mice with induced leucopenia have shown that even with bactericidal drugs the granulocytes assist in killing the organisms[162]. Nevertheless experimental *in vivo* studies in mice have shown interference by the tetracyclines with the action of penicillin[163,164,165], by the tetracyclines and chloramphenicol with the action of streptomycin. Caution, however, should be exercised in assuming that these conditions apply in natural infections, for although the type of interference, whether antagonism, synergism or indifference cannot be changed *in vitro* by altering the concentrations of the drugs[166], there is evidence that it can be changed *in vivo*; in experiments in mice infected with pneumococci an additive or antagonistic effect was obtained with chlortetracycline and penicillin simply by altering the doses of the drugs[167].

Conflicting differences between interference in drugs when tested under experimental conditions and when used in clinical practice occurs with the antitubercular drugs. *In vitro*[168] and in mice[169], streptomycin in sub-inhibitory concentrations antagonises the effect of isoniazid, yet this combination of drugs is very effective in man, where the concentrations of the drugs must be constantly changing.

To obtain definite clinical evidence of interference between drugs is not easy except by statistical methods but what undoubtedly appears to be antagonism between penicillin and chlortetracycline was observed in a series of cases of patients with pneumococcal meningitis. Intramuscular penicillin alone and intramuscular penicillin plus oral chlortetracycline were given in alternate cases, and of 14 patients treated with penicillin only 3 died but 11 died out of 14 treated with both drugs[170]. Also, in a series of patients with meningococcal meningitis penicillin alone was superior to a combination of it and chlortetracycline[171]. A study of the combination of penicillin and chloramphenicol in streptococcal pharyngitis revealed, however, no evidence of interference[172]. It has been queried[173] whether synergistic effects are required for any infection other than *Streptococcus fæcalis* endocarditis. The infection is apparently unique for the bactericidal effect of penicillin even in optimum concentrations is not complete against this particular streptococcus unless streptomycin is also present and in order to ensure a permanent cure every organism on the heart valve must be killed. It therefore seems unlikely that similar conditions exist in any other infection.

Unfortunately combinations of drugs do not show synergism to organisms that are resistant to one of the drugs, and even when the resistance is only partial the synergism may only be demonstrated *in vitro* with a concentration of the drug unobtainable *in vivo*[166].

The use of combinations of drugs in order to cover a wider range of bacterial species is probably rarely necessary with the wide-spectrum antibiotics available, but there may still be conditions in which these antibiotics are less suitable than a combination of other drugs. Penicillin

and streptomycin are very effective for the treatment of peritonitis[12,27]. The polymyxins and bacitracin have relatively narrow ranges of antibacterial activity but their activities are complementary. They are not absorbed from the gastro-intestinal tract and are therefore of value for specifically reducing the bacterial flora of the bowel, and as they rarely cause hypersensitivity they are very useful for topical application.

As one authority[173] points out two or more drugs are often given to an acutely ill patient suffering from an infection when a bacteriological or even clinical diagnosis has not been made, but the genuine necessity for this must be rare. The main objection to this form of treatment is that if commenced before the necessary pathological specimens are taken the diagnosis may be obscured. Sympathy is, however, felt for the practitioner who is fairly certain of his clinical diagnosis but is in doubt about the sensitivity of the causal organisms to the chemotherapeutic drugs and uses two or more of these. Sensitivity tests would disappear if a drug active against all organisms became available, and combined therapy is frequently no more than an attempt to anticipate this drug.

## References


1. Norris, *Brit. J. soc. Med.*, 1953, **7**, 112.
2. Medical Research Council, *Lancet*, 1953, **265**, 1163.
3. Leading article, *ibid.*, 1953, **265**, 1245.
4. Smythe, *ibid.*, 1948, **255**, 485.
5. Spink, Hall and Shaffer, *J. Amer. med. Ass.*, 1949, **139**, 352.
6. Poth, *ibid.*, 1953, **153**, 1516.
7. Kern and Wimberley, *Amer. J. med. Sci.*, 1953, **226**, 357.
8. Boger, Sherman, Schiller, Siegal and Rose, *J. Allergy*, 1953, **24**, 383.
9. Leading article, *Lancet*, 1954, **266**, 31.
10. Ministry of Health, *ibid.*, 1953, **265**, 33.
11. Crofton, *Brit. med. J.*, 1953, **2**, 1014.
12. Finland, *ibid.*, 1953, **2**, 1115.
13. Elias, Price and Merrion, *Antibiot. and Chemother.*, 1951, **1**, 491.
14. Welch, Randall and Hendricks, *ibid.*, 1953, **3**, 1053.
15. Fletcher and Knappett, *Brit. med. J.*, 1953, **1**, 188.
16. Walker and Hamburger, *Antibiot. and Chemother.*, 1954, **4**, 76.
17. Anderson and Landsman, *Brit. med. J.*, 1947, **2**, 950.
18. Doxiadis, Emery and Stewart, *ibid.*, 1951, **1**, 16.
19. Cathie and Macfarlane, *ibid.*, 1953, **2**, 805.
20. Foltz and Schimmel, *Antibiot. and Chemother.*, 1953, **3**, 593.
21. Fairbrother and Daber, *Lancet*, 1954, **266**, 858.
22. Lozinski and Gleason, *Brit. med. J.*, 1953, **2**, 150.
23. Jensen, Dragstedt, Kiaer, Nielson and Frederiksen, *Acta path. microbiol. scand.*, 1951, **28**, 407.
24. Ungar and Muggleton, *Brit. med. J.*, 1952, **1**, 1211.
25. Gylee, Bayne and Boger, *Antibiot. and Chemother.*, 1954, **4**, 133.
26. Engelbreth-Holm and Roholt, *Acta pharm. tox. Kbh.*, 1953, **9**, 117.
27. Meleney and Johnson, *J. Amer. med. Ass.*, 1953, **153**, 1253.
28. Rountree and Thompson, *Lancet*, 1952, **263**, 262.
29. Kirby and Ahern, *Antibiot. and Chemother.*, 1953, **3**, 831.
30. Garrod, Report of Meeting Royal Society of Medicine, *Lancet*, 1952, **262**, 400.
31. Forbes, *Brit. med. J.*, 1953, **1**, 1139.
32. Garrod, *ibid.*, 1950, **2**, 722.
33. Thomson, *J. clin. Path.*, 1952, **5**, 169.
34. Medical Research Council, *Lancet*, 1953, **264**, 1109.
35. Lewis, Putnam, Hendricks, Kerlan and Welch, *Antibiot. and Chemother.*, 1952, **2**, 601.
36. Hodgkinson, *Lancet*, 1954, **266**, 285.
37. Gocke and Finland, *J. Lab. clin. Med.*, 1951, **38**, 719.
38. Editorial, *Antibiot. and Chemother.*, 1953, **3**, 659.

39. Kirby, *J. Amer. med. Ass.*, 1950, **144**, 233.
40. Sayer, Michel, Moll and Kirby, *Amer. J. med. Sci.*, 1951, **221**, 263.
41. McVay and Sprunt, *Ann. intern. Med.*, 1953, **38**, 955.
42. Montmorency, Caffery and Musselman, *Antibiot. and Chemother.*, 1954, **4**, 313.
43. Spink, Braude, Castaneda and Goytia, *J. Amer. med. Ass.*, 1948, **138**, 1145.
44. Harkness, *Brit. J. vener. Dis.*, 1953, **29**, 134.
45. Brown, Propp, Guest, Beebe and Early, *J. Amer. med. Ass.*, 1953, **152**, 2067.
46. Sharp, *Lancet*, 1954, **266**, 390.
47. Wilcox, *ibid.*, 1951, **261**, 154.
48. Seligmann, *Proc. Soc. exp. Biol. N.Y.*, 1952, **79**, 481.
49. Huppert, MacPherson and Cazin, *J. Bact.*, 1953, **65**, 171.
50. Leading article, *Lancet*, 1953, **265**, 1245.
51. Putnam, Hendricks and Welch, *Antibiot. and Chemother.*, 1953, **3**, 1183.
52. Report on Washington Symposium on Antibiotics, *Brit. med. J.*, 1953, **2**, 1043.
53. Finland, Purcell, Wright, Love, Mou and Kass, *J. Amer. med. Ass.*, 1954, **154**, 561.
54. Haight and Finland, *Proc. Soc. exp. Biol. N.Y.*, 1952, **81**, 183.
55. Lepper and colleagues. Report to Washington Symposium on Antibiotics, *Brit. med. J.*, 1953, **2**, 1043.
56. Hobson, *ibid.*, 1954, **2**, 236.
57. Haight, *J. Lab. clin. Med.*, 1954, **43**, 15.
58. Wong, James and Finlay, *Antibiot. and Chemother.*, 1953, **3**, 741.
59. Trafton and Lind, *Antibiot. and Chemother.*, 1954, **4**, 43.
60. Annotation, *Lancet*, 1953, **265**, 873.
61. Meleney and Johnson, *J. Amer. med. Ass.*, 1949, **7**, 794.
62. Brownlee, Bushby and Short, *Brit. J. Pharmacol.*, 1952, **7**, 170.
63. Swift and Bushby, *Lancet*, 1953, **264**, 110.
64. Swift and Bushby, *ibid.*, 1951, **261**, 183.
65. Batty Shaw and MacKay, Personal Communication, 1948.
66. Schoenbach, *J. Mt. Sinai Hosp.*, 1949, **16**, 71.
67. Jawetz, *Arch. intern. Med.*, 1952, **89**, 90.
68. Hayes and Yow, *Amer. J. med. Sci.*, 1950, **220**, 633.
69. Jackson, Lowbury and Topley, *Lancet*, 1951, **261**, 137.
70. Ainslie, *Brit. J. Opthal*, 1953, **37**, 336.
71. Jawetz, *Calif. Med.*, 1953, **79**, 99.
72. Kile, Rockwell and Schwarz, *Amer. med. Ass. Arch. Derm. and Syph.*, 1953, **68**, 296.
73. Waksman, Hutchison and Katz, *Amer. Rev. Tuberc.*, 1948, **60**, 78.
74. D'Esopo, *J. Amer. med. Ass.*, 1954, **154**, 52.
75. Dearing and Needham, *Proc. Mayo Clin.*, 1953, **28**, 507.
76. Wright, Purcell, Wilcox, Broderick and Finland, *J. Lab. clin. Med.*, 1953, **42**, 877.
77. Church, *Brit. med. J.*, 1954, **1**, 314.
78. Rist, *C. R. Soc. Biol. Paris*, 1939, **130**, 972.
79. Feldman, Hinshaw and Moses, *Proc. Mayo Clin.*, 1940, **15**, 697.
80. Anderson and Strachan, *Lancet*, 1948, **255**, 135.
81. Madigan, *ibid.*, 1948, **255**, 174.
82. Feldman, Hinshaw and Mann, *Amer. Rev. Tuberc.*, 1945, **52**, 269.
83. Youmans and MacCarter, *ibid.*, 1946, **52**, 432.
84. Hinshaw, Feldman and Pfeutze, *ibid.*, 1946, **54**, 191.
85. Krafchik, *J. Amer. med. Ass.*, 1946, **132**, 375.
86. Medical Research Council, *Brit. med. J.*, 1950, **2**, 1085.
87. Davis, Anderson and Landy, *Ann. west. Med. Surg.*, 1948, **2**, 245.
88. Edison, Frost, Graessle, Hawkins, Kuna, Muschett, Silber and Solotorovsky, *Amer. Rev. Tuberc.*, 1948, **58**, 487.
89. Riley, *Amer. J. med. Sci.*, 1953, **226**, 552.
90. Maher-Loughnan, *Tubercle Lond.*, 1952, **33**, 342.
91. Crofton and Mitchison, *Brit. med. J.*, 1948, **2**, 1009.
92. Daniels and Hill, *ibid.*, 1953, **1**, 1162.
93. Lehmann, *Lancet*, 1946, **250**, 15.
94. Feldman, Karlson and Hinshaw, *Proc. Mayo Clin.*, 1947, **22**, 473.
95. Swedish National Association against Tuberculosis, *Amer. Rev. Tuberc.*, 1950, **61**, 597.
96. Bogen, Loomis and Will, *ibid.*, 1950, **61**, 226.
97. Carstenen, *ibid.*, 1950, **61**, 613.

98. Russell, Jr., *Dis. Chest*, 1952, **21**, 439.
99. Bloch, Vennesland, Ebert and Gomori, *Amer. Rev. Tuberc.*, 1949, **59**, 554.
100. Hobby, Lenert, Rivoire, Domikian and Pikula, *ibid.*, 1953, **67**, 808.
101. Domagk, *ibid.*, 1950, **61**, 8.
102. Mertens and Bunge, *ibid.*, 1950, **61**, 20.
103. Hinshaw and McDermott, *ibid.*, 1950, **61**, 145.
104. Colwell, Moravec, Furutani and Ballard, *J. Lab. clin. Med.*, 1952, **39**, 761.
105. Muschenheim, Elmendorf, Jr., Cawthon and McDermott, *Amer. Rev. Tuberc.*, 1953, **68**, 791.
106. Bernstein, Lott, Steinberg and Yale, *ibid.*, 1952, **65**, 357.
107. Steenken, Jr. and Wolinsky, *ibid.*, 1952, **65**, 365.
108. Offe, Stiefken and Domagk, *Naturwissenschaften*, 1952, **39**, 118.
109. McVay and Sprunt, *J. Amer. med. Ass.*, 1953, **153**, 95.
110. Robitzek and Selikoff, *Amer. Rev. Tuberc.*, 1952, **65**, 402.
111. Steenken, Meade, Wolinsky and Coates, *J. Amer. med. Ass.*, 1952, **149**, 187.
112. Medical Research Council, *Brit. med. J.*, 1952, **2**, 775.
113. Steenken and Wolinsky, *Amer. Rev. Tuberc.*, 1953, **68**, 548.
114. Bloch, Widelock and Peizer, *ibid.*, 1953, **68**, 734.
115. Barnett, Bushby and Mitchison, *Brit. J. exp. Path.*, 1953, **34**, 568.
116. Mitchison, *Brit. med. J.*, 1954, **1**, 128.
117. Medical Research Council, *Lancet*, 1953, **265**, 213.
118. Medical Research Council, *Brit. med. J.*, 1953, **2**, 1005.
119. Anderson, Kerr and Landsman, *Lancet*, 1953, **265**, 691.
120. Clark, Elmendorf, Cawthon, Muschenheim and McDermott, *Amer. Rev. Tuberc.*, 1952, **66**, 391.
121. Garrod, *ibid.*, 1950, **62**, 582.
122. Mackaness and Smith, *ibid.*, 1953, **67**, 322.
123. Ritchie, Taylor and Dick, *Lancet*, 1953, **265**, 419.
124. Suter, *Amer. Rev. Tuberc.*, 1952, **65**, 775.
125 Barclay, Ebert, LeRoy, Manthei and Roth, *J. Amer. med. Ass.*, 1953, **151**, 1384.
126 Mackaness and Smith, *Amer. Rev. Tuberc.*, 1952, **66**, 125.
127. Singh and Mitchison, *Brit. med. J.*, 1954, **1**, 130.
128. United States Public Health Service Report, *Amer. Rev. Tuberc.*, 1954, **69**, 1.
129. Bernstein, Jambor, Lott, Pansy, Steinberg and Yale, *ibid.*, 1953, **67**, 357.
130. Dessau, Yeager, Burger and Williams, *ibid.*, 1952, **65**, 519.
131. Yeager, Munroe and Dessau, *ibid.*, 1952, **65**, 523.
132. McDermott, Ormond, Muschenheim, Deuschle, McCune, Jnr. and Tompsett, *ibid.*, 1954, **69**, 319.
133. Campagna, Calix and Hansen, *ibid.*, 1954, **69**, 334.
134. Lattimer, Lerman, Lerman. and Spivack, *J. Urol.*, 1953, **69**, 745.
135. Des Autels and Pfuetze, *Amer. Rev. Tuberc.*, 1953, **68**, 912.
136. Torres-Gost, *Lancet*, 1953, **265**, 693.
137. Fouqet, Heimann, Teyssier, Gibelin and Cornu, *Rev. Tuberc. (Paris)*, 1953, **17**, 30.
138. Fletcher, *Lancet*, 1953. **265**, 694.
139. Kerr, *Brit. med. J.*, 1953, **2**, 1130.
140. Levinson, *Amer. J. Dis. Child.*, 1949, **77**, 709.
141. Hobby, Lenert, Donikian and Pikula, *Amer. Rev. Tuberc.*, 1951, **63**, 434.
142. *Trans. 10th Conf. Chemother. Tuberc., V.A.*, 1951.
143. Millar, Sands, Walker, Dye and Tempel, *Amer. Rev. Tuberc.*, 1952, **66**, 534.
144. Rothstein and Johnson, *ibid.*, 1954, **69**, 65.
145. Millar, Sands, Gregory, Hightower, Weiser and Tempel, *ibid.*, 1954, **69**, 58.
146. Bartz, Ehrlich, Mold, Penner and Smith, *ibid.*, 1951, **63**, 4.
147. Steeken and Wolinsky, *ibid.*, 1951, **63**, 30.
148. Finlay, Hobby, Hochstein, Lees, Lenert, Means, P'An, Regna, Routien, Sobin, Tate and Kane, *ibid.*, 1951, **63**, 1.
149. Werner, Tompsett, Muschenheim and McDermott, *ibid.*, 1951, **63**, 49.
150. *Trans. 12th Conf. Tuberc., V.A.W.*, Feb. 1953.
151. Francis and Spinks, *Brit. J. Pharmacol.*, 1950, **5**, 565.
152. Cochrane, *Practitioner*, 1951, **166**, 373.
153. Bushby and Woiwod (to be published).
154. Cochrane, *Brit. med. J.*, 1952, **2**, 1220.
155. Erickson, *Int. J. Leprosy*, 1951, **19**, 1.
156. Lowe, *Lancet*, 1952, **260**, 436.
157. Report Sixth International Congress of Leprosy, 1953, *Int. J. Leprosy*, **21**, 477.

158. Bushby and Barnett, *ibid.*, 1953, **21**, 467.
159. Jawetz and Gunnison, *Antibiot. and Chemother.*, 1952, **2**, 243.
160. Flippin and Eisenberg, *Amer. J. med. Sci.*, 1954, **227**, 117.
161. Elek, Hillson and Jewell, *Brit. med. J.*, 1953, **2**, 1298.
162. Gowans, *Brit. J. exp. Path.*, 1953, **34**, 195.
163. Speck, Jawetz and Gunnison, *Arch. intern. Med.*, 1951, **88**, 168.
164. Bartell, *J. Inf. Dis.*, 1952, **90**, 273.
165. Jawetz, Gunnison and Speck, *Amer. J. med. Sci.*, 1951, **222**, 404.
166. Gunnison, Shevky, Bruff, Coleman and Jawetz, *J. Bact.*, 1953, **66**, 150.
167. Lepper, Jackson, Dowling and Seto, *J. Amer. med. Sci.*, 1953, **225**, 648.
168. Szybalski and Bryson, *Amer. Rev. Tuberc.*, 1953, **68**, 280.
169. Grunberg and Schnitzer, *ibid.*, 1953, **68**, 277.
170. Lepper and Dowling, *Arch. intern. Med.*, 1951, **88**, 489.
171. Lepper, Dowling, Werhlie, Blatt, Spies and Brown, *J. Lab. clin. Med.*, 1952, **40**, 891.
172. Walker, *Antibiot. and Chemother.*, 1953, **3**, 677.
173. Garrod, *Brit. med. J.*, 1953, **1**, 953.

# RESEARCH PAPERS

## AN *IN VIVO* METHOD FOR THE ASSAY OF HEPARIN

By J. Erik Jorpes, Margareta Blombäck and Birger Blombäck

*From the Chemistry Department II, Karolinska Institutet, Stockholm, Sweden*

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With the increasing use of heparin, the question of its assay has gained in importance. The matter has recently become topical, owing to the difficulties encountered in determining the anticoagulant activity of heparin. It has been found that the results obtained are greatly dependent on the methods applied, which is a serious drawback in a biological assay.

In a recent study[1], we pointed out that commercial samples of heparin assayed by the U.S.P. XIV method tended to give lower figures for the anticoagulant activity than when assayed with fresh whole blood or by a thrombin method. The discrepancy was, in fact, remarkable. Commercial samples of the Swedish heparin (Vitrum), when assayed by the U.S.P. method, frequently gave 20 per cent. lower figures than with the fresh whole blood method. A sample of the Danish heparin (Novo) analysed in 1953 showed an activity of 3335 I.U./mg. as assayed against the International Heparin Standard, instead of the declared strength of 5000 I.U./mg. The discrepancy was certainly not due to a deficiency in the strength of the heparin samples. Since the U.S.P. XIV method will be automatically accepted in most countries, the question of its reliability is of considerable importance.

In our previous communication[1], we reported on the analyses of 20 samples of heparin sodium, ranging in strength from 25 to 130 I.U./mg., performed by 4 different methods. They were: a fresh whole blood method, a thrombin method on plasma, the U.S.P. XIV method and the B.P. 1953 method. In samples with a strength of 25 to 110 I.U./m.g., the U.S.P. XIV method yielded 10 to 15 per cent. lower figures than the thrombin method. A striking deviation was noted in assays of the old Swedish standard heparin (see Table I).

TABLE I

Assay of the old Swedish standard heparin against the international standard heparin

| Whole blood method of Jalling, Jorpes and Lindén[2] | Thrombin method of Studer and Winterstein[3] | Plasma method of the U.S.P. XIV, 1950 |
|---|---|---|
| I.U./mg. | I.U./mg. | I.U./mg. |
| 80 | 83 | 65 |
| 80 | 79 | 68 |
| 85 | 86 | 70 |
| 82 | 83 | 70 |
| 80* (25.11.51) | 83 | 65 |
| 81** (23.7.52) | 78 | 65 |
| 76** (6.5.52) | — | 68 |
| 81 | 82 | 67 |

* Checked against Liquemin (Roche). ** Checked against Heparin (Novo).

It is seen from Table I that the Swedish standard heparin had a potency of 81 I.U./mg. when assayed by the whole blood method, whereas it was 82 I.U./mg. by the thrombin method of Studer and Winterstein. The U.S.P. method gave, however, only 67 I.U./mg. All the figures recorded in the Table I were obtained on different days.

In view of the difficulties of obtaining uniform results by the use of one or other of the *in vitro* methods, we concentrated our efforts on devising an *in vivo* method. There is every reason to attempt to dispense with the artificial systems hitherto in use, since they imply a lack of one or more of the normal coagulation factors, or the introduction of seriously disturbing components, such as an excess of thrombokinase or an unbalanced quantity of calcium.

Howell[4] preferred to use fresh cat blood, to which heparin was added *in vitro*. His method was improved and described in detail by Scott and Charles[5] and by Jaques and Charles[6]. One of us (E. J.) found ox blood to be preferable[7]. For the sake of convenience, however, artificial systems have been considered more advantageous. Among them are those with citrated plasma (U.S.P. XIV), with oxalated or citrated plasma and thrombin (Studer and Winterstein), and with salted whole blood (B.P. 1953).

As already pointed out, it seems to be a hopeless task to ascertain which of the aforementioned systems is to be regarded as the method of choice. Consequently, we have attempted to determine the anticoagulant activity in the only physiological way, *i.e.*, by injecting it intravenously into living animals, and determining the coagulation time a few minutes later. This technique ensures the presence of all the factors entering into the coagulation system, and no disturbing elements are introduced. Since we found that unanæsthetised sheep could be used, the influence of narcosis could also be eliminated.

Our experience with the *in vivo* technique in animals has hitherto been favourable. Moreover, we have been able to confirm that the methods using fresh, unaltered whole blood are fully reliable.

## Experimental

Our first experiments were made on cats and dogs under nembutal anæsthesia. Heparin of known strength was injected intravenously in small doses, increasing in size by 10 to 20 per cent., at intervals of 3 to 4 hours. The coagulation time of the blood, taken from the femoral vein, was determined 4 minutes after each injection. The values were plotted on a curve, from which the strength of an unknown sample could be read off after injection of a suitable dose. It soon became evident that application of this principle was feasible. Still better results were, however, obtained in sheep, since general anæsthesia was unnecessary and capillary bleeding could be avoided.

### *Technique*

The wool is cut, and the area over a jugular vein is anæsthetised by injecting 5 to 10 ml. of a 0·3 per cent. solution of lidocaine. In order to support the polyethylene tubing to be inserted into the jugular vein, a

specially constructed coupler—a cannulated plate as used by Hallgren and Björck[8] (see Fig. 1)—is fixed to the skin by means of two safety-pins. The plate (A. B. Jacoby, Stockholm) is provided with an elongated nozzle at one end and a Record fitting at the other; in the centre it has a stopcock.

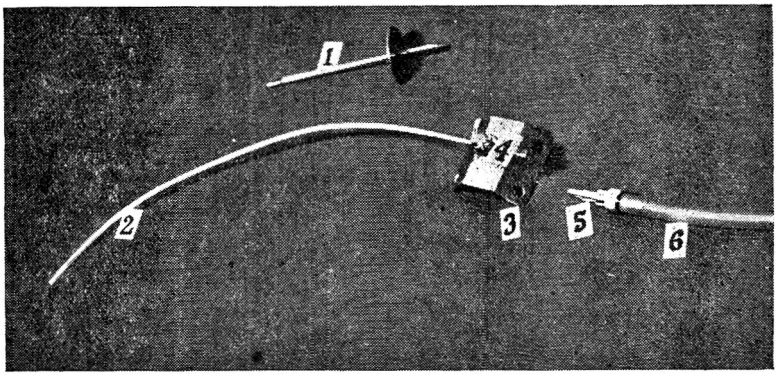


FIG. 1. The polyethylene tubing (2) fixed to the supporting plate (3).

The polyethylene tubing is drawn over the nozzle, and the rubber tubing attached to a drip flask containing physiological saline solution is connected to the Record fitting.

FIG. 2. The polyethylene tubing *in situ*.

A metal cannula with an inner diameter of slightly over 2 mm. is inserted into the jugular vein, and through it is passed a plastic tube (Polyethylene Medical Tubing, inner diameter 1·57 mm., outer diameter 2·08 mm., Pe 205, Clay Adams Co., Inc., New York). The tube is 20 cm. long and 10 cm. of its length is inserted. A single suture is passed through the skin and around the tube; the free end of the tube is then drawn over the nozzle of the plate. The polyethylene tubing is firmly attached in this way and cannot be displaced by the movements of the animal. The rubber tubing (inner diameter 5 mm.) which connects the plate with the flask containing physiological saline solution is sufficiently long to permit freedom of movement; the animal is able to eat, drink and lie down unhindered throughout the experiment, which lasts for up to 20 hours. (Fig. 2.)

During the whole experiment, a continuous flow of sterile, physiological saline solution runs into the vein at a rate of 15 to 20 drops per minute. This is to prevent the formation of clots in the polyethylene tubing. The heparin is injected through the rubber tubing, close to the plate, and is flushed down with a little saline solution. The blood samples are withdrawn with a 10-ml. Record syringe. For this purpose, the saline drip is disconnected, the syringe is connected to the fitting on the plate and is filled rapidly, the time being recorded with a stopwatch. The saline drip is then reconnected without delay.

Only small doses of heparin are given. The maximal effect is reached 4 minutes after the injection (Fig. 3); consequently, all the samples for determination of the coagulation time should be taken exactly 4 minutes after injection as recorded with a stopwatch.

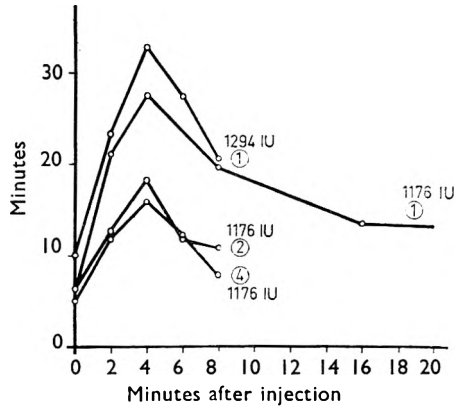


FIG. 3. The effect of small doses of heparin on the coagulation time at different times after injection.

We found a modified Bergquist chamber[9] to be convenient for the determinations of the coagulation time. The chamber, which is 17 by 6 by 4·6 cm., is made of transparent plastic and has a removable lid (Fig. 4). Moistened wads of cotton wool prevent the blood from drying. The floor of the chamber is elevated and has 3 holes, each of which contains a removable plastic cup. The upper surface of the cup is spherically concave, and on it is placed a watch-glass of standard size. A surface of plastic material is unsuitable. The cups, together with the watch-glasses, are rotated by means of a rod provided with cogs, which engage the cogs on the outer surface of the cups. 2 drops of blood are placed on each watch-glass with the Record syringe. When reading off, the chamber should be tilted at an angle 45° against a white background. Owing to the movement of the cups, the readings are fairly distinct.

2 chambers with 3 watch-glasses in each are used for each determination, thus giving 6 single determinations for each sample. In the individual sample, the normal values—i.e., 5 (4 to 6) minutes—usually deviate from each other by 15 to 20 seconds. With longer coagulation times, approaching 20 minutes, the readings are less distinct, and there is difficulty in fixing the end-value. If air bubbles or other impurities are present, the sample must be discarded. A single determination showed in the 10 to 15 minutes group (210 analyses) a spreading ($\sigma$) of 1·01 $\pm$ 0·05 minutes, in the 15 to 20 minutes group (157 analyses) 1·20 $\pm$ 0·07 minutes and in the 20 to 30 minutes group (74 analyses) 1·50 $\pm$ 0·12 minutes.

After injecting 800 to 1000 I.U. of heparin, an interval of about 4 hours must elapse before a new dose is given. The excretion apparently proceeded

more slowly at the end of an experiment, when the animal had been given, e.g., 4 injections. A similar delayed excretion has been reported by Merz[10] to occur in man, following the accumulation of repeated doses of heparin. In addition, the coagulation time must have returned to normal

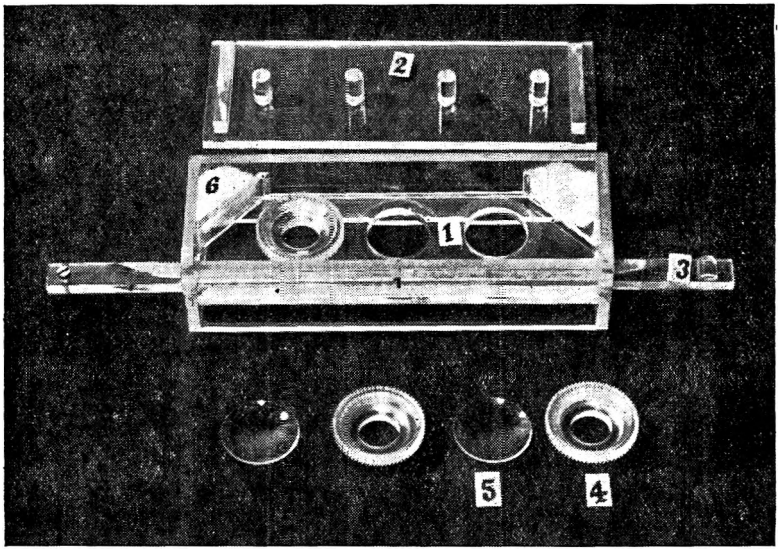


FIG. 4. The modified Bergqvist chamber for the determination of the coagulation time. The perforated floor (1), the lid (2) lying upside down, the rod keeping the cups rotating (3), a plastic cup (4), supporting the watch-glass (5), moistened cotton (6).

before administering a further dose. An elevation of only 1 minute necessitates a new test after $\frac{3}{4}$ to 1 hour. It is desirable to use 4 or 5 animals concurrently.

In our series of experiments, none of the animals suffered any ill effects. The only precaution taken was to cleanse the operation field with ethanol at the beginning of the procedure, as well as after removal of the polyethylene tubing, the suture around it and the metal plate. Finally, in order to lessen the risk of thrombosis, 50 mg. of heparin were injected at the end of the experiment.

## RESULTS

As seen in Figure 5, a difference of 10 per cent. in the quantity of heparin injected resulted in a distinct difference in the coagulation time. Consequently, it should be possible to locate the strength of an unknown sample of heparin between two points differing in strength by 10 per cent. In fact, this proved to be the case.

The first sample assayed was a new Swedish national standard heparin. Its strength had been thoroughly analysed with the fresh whole blood method[1,2] and with the thrombin method of Studer and Winterstein. The former method gave 108·6 I.U./mg. as assayed on 2 days against the old

Swedish standard heparin, and 108·1 I.U./mg. as assayed likewise on 2 days against the international standard heparin. On each of the 4 days, 15 or 18 stands were used. With the thrombin method, and assaying against the international standard heparin, the results on 3 different days were 104, 110 and 105 I.U./mg., respectively (mean: 107 I.U./mg.).

Of the sample assumed to contain 108 I.U./mg. doses of 600 to 1100 units were injected into 4 sheep and curves plotted. Then doses of 1000 and 660 I.U., respectively, of the international standard heparin were given to 2 of the animals. Found: 990 and 614 I.U. 2 doses of 1000 and 1100 I.U. respectively were given to a third animal. Found: 990 and 1025 I.U. Thus, the international standard heparin was 1·0, 7·0, 1·0 and 7·5 per cent., respectively (mean 4·1 per cent.) weaker than the sample used in plotting the curves.

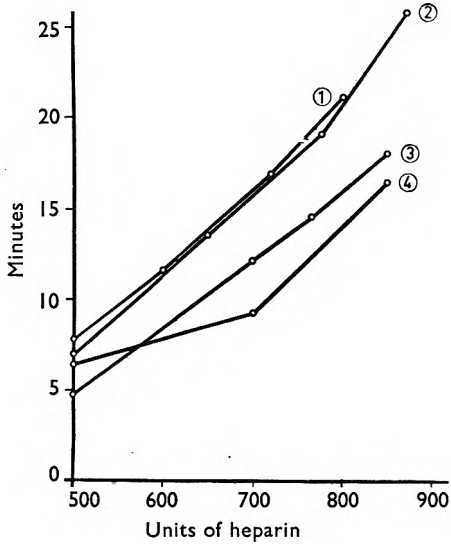


FIG. 5. The effect upon the coagulation time of injecting successively increasing small doses of heparin intravenously in sheep as measured 4 minutes after injection. 1, 2, 3, 4, different animals.

On another day, the international standard heparin was found to contain 123 and 139 I.U./mg. (mean 131 I.U./mg.) when assayed in the same way in 2 animals.

Consequently, the new Swedish national standard heparin contains 108 I.U./mg. of water-free substance as assayed with the fresh whole-blood method, 107 I.U./mg. when assayed with thrombin and citrated plasma, and approximately 110 I.U./mg. when assayed in living animals (sheep).

The next sample analysed was the old Swedish standard heparin, which had earlier been found to contain 81 I.U./mg. (Table I) with the fresh whole-blood method. 4 animals were given injections of the new national standard heparin (108 I.U./mg. of water-free substance) and curves were plotted. The figures found for the old standard heparin in 3 of the animals were 78·3, 80·5 and 82·1 I.U./mg. respectively (mean: 80·3 I.U./mg.). In the fourth animal, the previous dose of heparin had evidently not been completely eliminated when the unknown was given. It is remarkable how closely this figure is in agreement with the previous one, 81 I.U./mg., obtained earlier with the fresh whole-blood method (Table I). The figure found with the U.S.P. XIV method was 67 I.U./mg.

For the sake of comparison the new Swedish standard heparin was also assayed by the U.S.P. XIV method and by the B.P. 1953 method. The first gave on 2 different days with different samples of sheep plasma and 2

dilutions of each of the international standard heparin, and of the new Swedish standard heparin 94·4 and 98·0 (mean: 96·2) I.U./mg. of water-free substance. The B.P. 1953 method gave 106 I.U./mg. on one day and 100 I.U./mg. on another day.

With the B.P. 1953 method the old Swedish heparin standard was found to contain 70·7 I.U./mg. In a series of earlier experiments, performed on 7 different occasions during the course of one year, the mean figure 73 I.U./mg. had been found.

## Discussion

As pointed out in previous papers[1,2], fresh whole blood seems to be the most adequate menstruum for measuring the anticoagulant activity of heparin. In all the artificial systems, some factor is lacking or foreign influences are introduced, thus making it impossible to compare the respective methods. In fresh whole blood, both the heparin co-factor and other labile factors are present, and the salt and thromboplastin content is normal. The only unphysiological factor is the mixing of blood with the anticoagulant *in vitro*. Logically, the next step would be to inject heparin directly into the animal and to follow the changes in the coagulation time. The living animal does, in fact, react to increasing doses of heparin in a stoichiometric way. Consequently, the anticoagulant heparin can be assayed in the same way as the other hormones.

The artificial coagulation systems used in the assay of heparin are far from reliable[2]. Some of them, e.g. the recalcified sheep plasma used in the *U.S.P. XIV method*, may give 20 per cent. lower figures than the whole blood method and the *in vivo* technique. The peculiarities of this method are discussed in our earlier paper[2] (page 1039). Of all the methods suggested, this is the most difficult to handle and the least reliable.

The *thrombin method of Studer and Winterstein* has many advantages. It is easy to work with and gives usually a correct level for the strength. Nevertheless, it has its pitfalls. At one time, we consistently obtained values too high by 20 per cent. on analysing 16 different samples of heparin, until we found that a new batch of plasma gave the correct figures. The method can, in fact, give the most peculiar results. Thus, the $\beta$-heparin of Marbet and Winterstein[11,12] was found to contain about 50 (sheep) and 25 (ox) I.U./mg. With the whole blood method, the figures were 3 to 4 and 8 I.U./mg., respectively, and with the *in vivo* method 3 and 8 I.U./mg., respectively, as assayed in both the cat and the dog. The U.S.P. XIV method showed no anticoagulant activity whatsoever, or less than 2 to 3 I.U./mg. (Yamashina[13]).

*The B.P. 1953 method* worked out by Adams and Smith[14] is rapid and simple in performance. As pointed out by Smith the data can be submitted to analysis of variance and the fiducial limits can easily be obtained. Unfortunately, however, this tells very little about the anticoagulant activity, the heparin sample would exert under physiological conditions. In several instances we also found the level of strength indicated by this method to be erroneous. Thus a sample of Liquemin Roche, recently analysed by us, gave only 4480 I.U./ml. on one occasion and 4130 I.U./ml.

on another day instead of 5000 I.U./ml. It is evident that difficulties will arise, if this method alone is applied in assaying commercial samples of heparin.

Since the different methods for the assay of heparin, which utilise artificial coagulation systems, tend to give deviating and sometimes evidently erroneous results (see Table II), there is a need for a reliable technique. For this purpose it seems to be possible to use fresh whole blood *in vitro*. Judging by our experience the fresh whole-blood method, using ox blood, gives figures equivalent to those obtained in injecting heparin *in vivo* in the sheep.

TABLE II

THE STRENGTH OF THE NEW SWEDISH STANDARD HEPARIN (I) AND THE OLD SWEDISH STANDARD HEPARIN (II) AS FOUND IN USING DIFFERENT METHODS OF ASSAY. I.U./MG. WATER-FREE SUBSTANCE

| *In vivo* (sheep) | Fresh ox-blood (Jalling *et al.*[2]) | Thrombin method (Studer and Winterstein.) | U.S.P., 1950 | B.P., 1953 |
|---|---|---|---|---|
| I 110 | 108 | 107 | 96 | 103 |
| II 80 | 81 | 82 | 67 | 73 |

## SUMMARY

1. The anticoagulant activity of heparin has been assayed in sheep, by giving small consecutive doses intravenously at intervals of 4 hours, and determining the coagulation time 4 minutes after injection. A curve is plotted, using doses of a standard heparin varying in strength by 10 to 20 per cent. This is followed by 1 or 2 injections of the unknown heparin in suitable doses. The figures found agree, with a margin of a few per cent., with the theoretical ones.

2. The fresh whole-blood method, using ox blood *in vitro*, is found to give figures identical with those obtained with the *in vivo* method.

3. The unreliability of the methods using artificial systems with citrated plasma or salted whole blood, including the U.S.P. XIV method, is discussed. An *in vivo* method is devised as a reference technique in assaying heparin.

## REFERENCES

1. Blombäck, Blombäck, Corneliusson and Jorpes, *J. Pharm. Pharmacol.*, 1953, **5**, 1031.
2. Jalling, Jorpes and Lindén, *Quart. J. Pharm. Pharmacol.*, 1946, **19**, 96.
3. Studer and Winterstein, *Helv. Physiol. Pharmacol. Acta*, 1950, **9**, 6.
4. Howell, *Amer. J. Physiol.*, 1924, **71**, 553.
5. Charles and Scott, *J. biol. Chem.*, 1933, **102**, 425, 437.
6. Jacques and Charles, *Quart. J. Pharm. Pharmacol.*, 1941, **14**, 1.
7. Jorpes, *Biochem. J.*, 1935, **29**, 1817.
8. Hallgren and Björck, *Nord. Vet. Med.*, 1953, **5**, 1.
9. Bergqvist, *Acta chir. scand.*, 1945, **92**, Suppl. 101.
10. Merz, *Schweiz. med. Wschr.*, 1954, **84**, 315.
11. Marbet and Winterstein, *Helv. chim. Acta*, 1951, **34**, 2311.
12. Marbet and Winterstein, *Experientia*, 1952, **8**, 41.
13. Yamashina, *Acta chem. scand.*, 1954. In print.
14. Adams and Smith, *J. Pharm. Pharmacol.*, 1950, **2**, 836.

# ALKALOID BIOGENESIS

## PART III. THE PRODUCTION OF BIOSYNTHETIC RADIOACTIVE HYOSCINE AND METELOIDINE

BY W. C. EVANS and M. W. PARTRIDGE

*From the University, Nottingham*

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THE use of $^{14}C$ in experimental alkaloid chemistry has hitherto involved studies of two types. By growing plants in $^{14}CO_2$, radioactive nicotine[1,2,3], morphine[4], colchicine[5], cinchona alkaloids[6] and veratrum alkaloids[7], usually of high specific activity, have been produced. In the second series of experiments, specific labelled precursors of a fragment of the alkaloid molecule, especially the methyl group, have been employed in studies of the mechanisms of synthesis occurring in plants[8–17]. From this work, important deductions have been made relating to the origin of gramine, nicotine and hordenine and to methylation processes occurring in plants. In plants containing alkaloids of the tropane group, the observation[18] that *Atropa belladonna* does not furnish radioactive alkaloids from $^{14}C$-labelled putrescine has provided powerful evidence that this compound is not a precursor of the alkaloids[19].

This communication describes the production of radioactive hyoscine and meteloidine in *Datura ferox* after injection of glycine-2-$^{14}C$.

### EXPERIMENTAL

Vigorously growing plants of *Datura ferox* which had been raised under glass and planted out in the open in June were selected for study.

*Method of Injection.* Injection was carried out on a fine day. The main stem, beneath one of the dichasial branches, was slit longitudinally about 5 cm. and transversely to a depth of one-third to one-half the diameter of the stem in order to produce a flap joined to the stem at its upper end. About 3 mm. of the lower end of the flap was cut away and a glass tube was fixed in position with the tip of the flap touching the bottom of the tube. Immediately the tube was in position it was partly filled with water, plugged with cotton-wool and provided with a dust cap. At no stage was the cut tissue of the flap allowed to become dry. Capillary creep of the liquid from the tube down the cut stem did not occur provided that the tube was not too full and that the flap was directed into the tube at a wide angle. The plants were firmly supported with stakes. All plants to be injected were prepared in this manner.

For injection, the water was pipetted from the tube and replaced by the solution to be injected. Uptake was complete by evening when the injection solution was placed in the tube in the middle of the afternoon. As the tubes emptied, two successive quantities of water were added to complete the uptake of the injection solution. During the first day, the plants were covered with cloches when rain threatened. The doses administered varied from 15 to 50 $\mu$c. of glycine-2-$^{14}C$ in the form of a

0·12 per cent. solution in water; control plants received the same quantities of glycine.

*Autoradiographs.* After intervals of 1 to 44 days, 3 discs, each of 1·5 cm. diameter, were cut from the leaves on the side of the plant above the site of injection. The fresh and air-dry weight of the 3 discs were 0·1 g. and 0·02 g. respectively.

For extraction, the discs were macerated with 1 drop of dilute solution of ammonia and 3 to 4 ml. of ethanol; after 3 hours the menstruum was pipetted off and the maceration was repeated with a further quantity of ethanol. The leaf tissue rapidly became brittle and was powdered under the solvent with a glass rod. After evaporation of the solvent, the residue was disolved in 2 drops of ethanol and the solution was placed on a paper strip. The components of the mixture were separated chromatographically by capillary rise, the organic solvent layer from a mixture of light petroleum (b.pt. 60° to 80° C.) 1 volume, amyl alcohol, glacial acetic acid and water, of each, 3 volumes, being used for the development.

Organic solvents on the developed paper strip were allowed to evaporate spontaneously and autoradiographs were obtained using Ilford, Industrial G, X-ray film. The time of exposure was 2 to 4 weeks. In order to test for the absence of chemical fogging, the control paper chromatograms were submitted to the same treatment. After marking coloured and fluorescent zones, the paper strip was cut longitudinally in order to locate amino-acids by means of ninhydrin reagent and alkaloids by means of modified Dragendorff's reagent[20].

Chromatograms and autoradiographs were prepared from the dried plant material after harvesting. In order to concentrate the alkaloids in this material, 0·2 g. was macerated as described above and the extract was evaporated to dryness. A solution of the residue in 0·2N sulphuric acid was washed with successive quantities of chloroform until colouring matter had been removed; traces of alkaloid were recovered from the chloroform with 0·2N acid and then the alkaloids were liberated by ammonia and collected in chloroform. All the solutions were evaporated to a small volume and placed on paper strips for chromatography.

*Isolation of Radioactive Hyoscine and Meteloidine.* The plant material was powdered in a ball mill and mixed with one-tenth its weight of calcium hydroxide. Sufficient water was added to moisten the mixture and, after an hour, the alkaloids were extracted by repeated maceration with ether. The ether was evaporated and the alkaloids were fractionated chromatographically by the method we have previously described[21].

The solutions remaining after titration of the two alkaloids were faintly acidified, indicator was removed by washing with chloroform, and the alkaloids were precipitated as their picrates; these salts were recrystallised from water and characterised by their melting-points and mixed melting-points.

*Hydrolysis of the Alkaloids.* Hyoscine picrate (3 mg.) was heated with 2 ml. of solution of barium hydroxide (5 per cent.) in a sealed tube in a boiling water bath for 30 minutes. Oscine was collected in chloroform,

converted into its hydrochloride by the addition of ethanolic hydrogen chloride and the solution was evaporated to dryness on a counting planchet. For the recovery of tropic acid, the aqueous liquid was made acid with hydrochloric acid and extracted with chloroform. Triethylamine was added to convert the acid in the chloroform into its salt and the solution was evaporated on a planchet. Meteloidine was hydrolysed and tiglic acid recovered in a similar manner; the remaining mother liquor was evaporated to dryness and the residue was extracted with ethanol to recover teloidine hydrochloride.

In *ad hoc* experiments, it was shown that hyoscine and meteloidine were completely hydrolysed under the conditions described above.

*Isolation of Radioactive Calcium Oxalate.* A portion of the marc of the leaves remaining after the extraction of the alkaloids was macerated with 3 successive quantities of dilute hydrochloric acid. After treatment of the extract with charcoal, calcium oxalate was precipitated by the addition of solution of ammonia, collected and washed thoroughly. The oxalate was decomposed by acidified potassium permanganate and the liberated carbon dioxide was swept into solution of barium hydroxide and recovered as barium carbonate.

## Results

Not more than a trace of radioactive glycine was detected on the autoradiographs within one day of its injection but 6 different radioactive areas coincident with those responding to ninhydrin reagent were observed. Up to 9 days after injection, the number of active zones diminished and thereafter no significant change was detected; the greatest activity was associated with the green pigments and with a zone exhibiting a blue fluorescence. Radioactive amino-acids had disappeared. Active zones on the autoradiographs prepared from the seeds of a plant receiving 50 $\mu$c. of radioactivity occupied similar positions to some of those present in the leaf autoradiographs. No radioactive alkaloid was detected by autoradiography in these samples.

In spite of changes which occurred on drying the plants as demonstrated by changes in their ultra-violet fluorescence chromatograms, the dried plant appeared to contain the same radioactive compounds as the fresh plant. Radioactive amino-acids were not detected in the dried plant.

The autoradiographs of the chromatograms prepared from 0·2 g. of dried material from the plant receiving 50 $\mu$c. of radioactivity, exhibited radioactive zones coincident with the alkaloid zones on the chromatogram. Appreciably more radioactive material was present in the fraction containing the colouring matter and in the alkaline aqueous liquid remaining after removing the alkaloids. The latter solution contained amino-acids but none was radioactive.

In Table I values are given for the activities of the samples listed as determined by counting. The products of hydrolysis had the following activities in disintegrations per minute per milliatom of carbon: hyoscine, $1{\cdot}25 \times 10^5$; oscine, $1{\cdot}5 \times 10^5$; tropic acid, $2{\cdot}1 \times 10^4$; meteloidine,

$1{\cdot}15 \times 10^5$; teloidine, $1{\cdot}7 \times 10^5$; tiglic acid, $2{\cdot}6 \times 10^4$. The dried leaves of the plant receiving 50 $\mu$c. of radioactivity contained about 4 per cent. of calcium oxalate which had an activity of $9 \times 10^4$ disintegrations per minute per millatom of carbon.

TABLE I

| Sample | Dose $\mu$c. | Period between injection and harvest days | Radioactivity† Disintegrations per minute per milligram | | | | |
|---|---|---|---|---|---|---|---|
| | | | Powdered material | Exhausted material | Hyoscine picrate | Meteloidine picrate | Colouring matter |
| Plant A* | 50 | 44 | | | | | |
| (i) Leaves from above site of injection | | | $6 \times 10^3$ | $3{\cdot}5 \times 10^3$ | $4 \times 10^3$ | $3 \times 10^3$ | $6 \times 10^3$ |
| (ii) Leaves from side opposite to site of injection | | | $5 \times 10^2$ | — | $3 \times 10^3$ | $3 \times 10^3$ | — |
| (iii) Root | | | $2 \times 10^3$ | — | $4 \times 10^3$** | | — |
| Plant B, leaves from whole plant | 15 | 32 | $1{\cdot}5 \times 10^3$ | — | $3{\cdot}5 \times 10^3$ | $3{\cdot}5 \times 10^2$ | — |

* Dried material contained hyoscine, 0·11 per cent. and meteloidine 0·03 per cent.
** Mixed picrates of total bases.
† Corrected for background count and self-absorption.

## Discussion

The evidence of the autoradiographs showed that glycine was rapidly metabolised to other amino-acids or simple ethanol-soluble peptides. This was followed by a slower disappearance of all the radioactive materials giving a colour with ninhydrin. The appearance of radioactivity in fractions associated with the colouring matter and in calcium oxalate implies that a considerable proportion of the glycine entered the general metabolic processes of the plant.

The general distribution of radioactivity throughout the plant was by no means uniform. Of that injected, the greatest quantity remained in the organs immediately above the site of injection; some was translocated to the root and a still smaller proportion to the side of the plant opposite the site of injection.

Glycine did not appear to be a very efficient starting material for the biosynthesis of these alkaloids. Marked differences in the activity of the alkaloids formed in the more mature plant receiving 50 $\mu$c. and in the less mature plant receiving 15 $\mu$c. indicated that the state of maturity of the plant was of some significance. The distribution of radioactive alkaloids was fairly uniform although hyoscine, but not meteloidine, recovered from near the site of injection appeared to be more active than that occurring on the opposite side of the plant.

Radioactive carbon was not uniformly distributed throughout the alkaloid molecules. Glycine provided a considerably more efficient source of carbon for the tropane ring than for the tropic acid or tiglic acid.

We are greatly indebted to Mr. D. R. Healey, Boots Pure Drug Co. Ltd. for carrying out the determinations of radioactivity.

## Summary

1. The production of biosynthetic radioactive hyoscine and meteloidine in *Datura ferox* after injection of glycine-2-$^{14}$C is described.

2. The major part of the radioactive carbon is concentrated in the tropane residues.

## References

1. Ganz, Kelsey and Geiling, *Bot. Gaz.*, 1951, **113**, 195.
2. Kuzin and Merenova, *C.R. Acad. Sci. U.R.S.S.*, 1952, **85**, 393.
3. Geiling, Kelsey, McIntosh and Ganz, *Science*, 1948, **108**, 558.
4. McIntosh, Kelsey and Geiling, *J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 512.
5. Walaszek, Kelsey and Geiling, *Science*, 1952, **116**, 225.
6. de Moerloose and Ruyssen, *Pharm. Tijdschr. Belg.*, 1953, **30**, 97.
7. Pircio and Geiling, *J. Amer. pharm. Ass., Sci. Ed.*, 1952, **41**, 552.
8. Sribney and Kirkwood, *Nature, Lond.*, 1953, **171**, 931.
9. Bjerrum and Wing, *J. biol. Chem.*, 1953, **205**, 637.
10. Bowden, *Nature, Lond.*, 1953, **172**, 768.
11. Dawson, Christman and Anderson, *J. Amer. chem. Soc.*, 1953, **75**, 5114.
12. Kirkwood and Marion, *Can. J. Chem.*, 1951, **29**, 30.
13. Bowden and Marion, *ibid.*, 1951, **29**, 1037, 1042.
14. Leete, Kirkwood and Marion, *ibid.*, 1952, **30**, 749.
15. Leete and Marion, *ibid.*, 1953, **31**, 126, 1195.
16. Matchett, Marion and Kirkwood, *ibid.*, 1953, **31**, 488.
17. Dubeck and Kirkwood, *J. biol. Chem.*, 1952, **199**, 307.
18. Diaper, Kirkwood and Marion, *Can. J. Chem.*, 1951, **29**, 964.
19. Cromwell, *Biochem. J.*, 1943, **37**, 717, 722.
20. Machebouef and Munier, *Bull. Soc. Chim. biol., Paris*, 1949, **31**, 1144.
21. Evans and Partridge, *J. Pharm. Pharmacol.*, 1952, **4**, 769.

# PIPERAZINE ADIPATE: A NEW ANTHELMINTIC AGENT

## PART I. PHYSICOCHEMICAL PROPERTIES

BY M. T. DAVIES, J. FORREST, F. HARTLEY and V. PETROW

*From the Research Laboratories, The British Drug Houses Ltd., London, N.1*

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ALTHOUGH piperazine and its water-soluble salts have long been used medicinally in the treatment of gout and rheumatism because of their possible value in dissolving uric acid, the anthelmintic potentialities of piperazine and its derivatives have been realised only in recent years. Following the discovery of the value of diethylcarbamazine citrate[1] in the treatment of filariasis, attention has been turned to the evaluation of other piperazine derivatives against helminth infections. In 1951, Mouriquand, Roman and Coisnard[2] reported the successful use of piperazine hydrate in the treatment of threadworm infestation in children. Wider use of this preparation was not immediately forthcoming as piperazine hydrate is a strongly basic substance of uncertain keeping qualities in solution. A more satisfactory derivative of piperazine was studied the following year by Turpin, Cavier and Savaton-Pillet[3], who described the employment of the bisphenylacetate salt, which was administered orally with concomitant use of piperazine suppositories.

Piperazine bisphenylacetate, though representing an advance on piperazine hydrate from the standpoint of pharmaceutical presentation, was not considered by us a satisfactory salt for the following reasons: (i) it contains only 25 per cent. of piperazine, so that the dose required is large, (ii) the material has a distinct urinary odour which renders it unpalatable to many patients and (iii) its solubility characteristics present difficulty in its alternative formulation as a liquid preparation stable to storage over a wide temperature range. We consequently turned our attention to other salts of piperazine. In particular, we sought a derivative of low solubility as we thought that such a compound might undergo slower absorption in the gut than the readily soluble hydrate, citrate, etc., thus avoiding the neurotoxic effects accompanying the use of the more soluble piperazine compounds which have subsequently been discussed[4,5]. The search for such a compound proved difficult, in that with one exception, the sparingly soluble salts prepared had either too high a molecular weight to offer advantages over the bisphenyl acetate or else were too toxic for oral administration. Piperazine adipate[6] alone of the many salts examined proved able to satisfy the pharmaceutical requirements we considered necessary. It was consequently submitted to pharmacological investigation (Part II) which showed it to be a relatively non-toxic compound with a high margin of safety. Preliminary clinical trials[7,8] showed that piperazine adipate achieved complete eradication of threadworm infestation in man without the occurrence of any side effects and wider clinical usage has confirmed these qualities[9]. The salt was also examined, at our request, by The Cooper Technical Bureau for its

veterinary applications. This study[10] showed that piperazine adipate was an effective curative agent against a variety of helminth infections in domestic animals.

The main advantages of piperazine adipate over the hydrate and its more soluble salts, are (i) its anthelminitic effect, which is markedly greater than that produced by an equivalent weight of piperazine as hydrate (ii) its freedom from side effects in clinical use (iii) its stability on storage, (iv) its high piperazine content and (v) its pleasant acidulous taste and freedom from odour.

Piperazine adipate[6], the neutral salt of piperazine and adipic acid $C_4H_{10}N_2 \cdot C_6H_{10}O_4$, forms colourless prisms of melting point 256° to 257° C. (uncorr.), which are characterised by a defect insertion of minute crystals. It remains unchanged on exposure to air and even after heating at 100° C. for prolonged periods its melting point and characteristics remain unchanged. It is non-hygroscopic and separates from aqueous solutions without water of crystallisation.

Piperazine adipate dissolves rather slowly in water at room temperature and is soluble to the extent of only about 5 per cent., the temperature gradient of solubility being shown in Table I. It is essentially insoluble in the lower aliphatic alcohols (see Table I).

TABLE I

| Solvent | Temp. ° C. (± 0·2° C.) | Solubility g./100 g. of solvent | Solvent | Temp. ° C. (± 0·2° C.) | s |
|---|---|---|---|---|---|
| Water | 20·0 | 5·53 | Methanol .. .. .. | 25·0 | 0·02 |
| | 25·0 | 6·02 | Ethanol (aq. 44 per cent.) .. | 25·0 | 0·57 |
| | 30·0 | 6·61 | Ethanol (99·5 per cent.) .. | 25·0 | very low |
| | 37·0 | 7·49 | *iso*Propanol .. .. .. | 25·0 | " |
| | 46·0 | 8·65 | Dioxan .. .. .. .. | 25·0 | " |
| | 56·3 | 10·14 | | | |

Aqueous solutions have *p*H 5·45 at 25° C. over the concentration range of 0·2 to 0·01M (i.e., 0·23 to 4·6 per cent. w/v) and this value is only slightly affected by increases in ionic strength caused by addition of simple neutral salts.

The classical dissociation exponent ($pK'_a$) of piperazine has previously been determined by three groups of workers[11,12,13]. The thermodynamic exponents ($pK_a$ at 25° C.), however, are only reported by Smith and Smith. We have, consequently, determined these constants *de novo*, the values obtained being given in Table II.

TABLE II

THE DISSOCIATION EXPONENTS OF PIPERAZINE AND OF ADIPIC ACID IN AQUEOUS SOLUTION

| Compound | Ref. | Temp. ° C. | $pKa_1$ | $pKa_2$ |
|---|---|---|---|---|
| Piperazine .. | 11 | 15 | 4·05† | 8·34† |
| | 12 | 25 | — | 9·8† |
| | 13 | 25 | 5·32 | 9·70 |
| | * | 25 | 5·29 | 9·66 |
| Adipic acid .. | 14 | 25 | 4·43 | 5·41 |

* Current investigation. † Classical $pK_a$ values only.

Consideration of this Table shows that, in solution, piperazine adipate should behave as a very weak monobasic acid and this is indeed found to be the case. Thus potentiometric titration with sodium hydroxide gives the sigmoid curve A (Fig. 1) normally shown by compounds of this type. Addition of a slight excess of a strong acid leads to complete ionisation of the piperazine with concomitant suppression of the ionisation of the adipic acid. Titration with alkali now gives rise to curve B (Fig. 1)

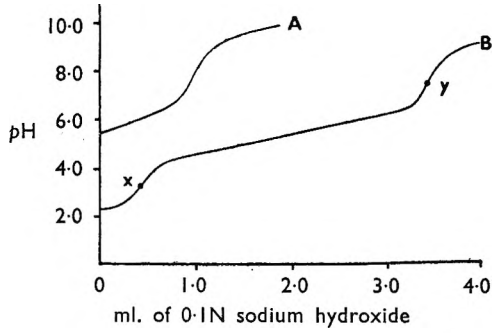


FIG. 1. Titration of 10 ml. aliquots of 0·01M piperazine adipate solution by standard alkali.
A. In the absence of added mineral acid
B. In the presence of 2·5 ml. of 0·1N hydrochloric acid.

in which the difference of titre corresponding to the two points of inflection x and y is exactly 3 times the titre of the original solution (*cf.* curve A). Piperazine adipate may consequently be readily estimated in this way, the tiration being unaffected by such electrolytic species as ammonium ions, alkali metal ions, or alternatively by the presence of weak acids of $pK_a < 4$ or of strong mineral acids. Extension of the method to the determination of piperazine in urine is under investigation.

## EXPERIMENTAL

### *Practical notes*

Potentiometric titrations were effected by means of a Muirhead direct-reading *p*H meter, with glass-calomel electrode assemblage incorporating a 3·5N potassium chloride bridge and capillary liquid junction. The linearity of response of the meter was checked by comparison with a standard potentiometer in order to ensure an instrumental accuracy of $\pm$ 0·01 *p*H unit. Alignment of the meter was carried out by means of 0·05M solutions of potassium hydrogen phthalate and sodium borate (borax), of *p*H 4·01 and 9·18, respectively, at 25° C. These solutions were prepared from the recrystallised A.R. salts, and accord with British Standard Specification 1647: 1950. Piperazine hexahydrate and adipate were prepared from commercial and laboratory samples, respectively, by recrystallisation from water, the adipate to constant melting point after drying over phosphorous pentoxide. Solutions of the hexahydrate were

prepared as required, using carbon dioxide free water. Their concentrations were determined by titration against standard 0·1N hydrochloric acid, using bromophenol blue as indicator. B.D.H. volumetric solutions of 0·1N hydrochloric acid were employed as the standard acid. Solutions of sodium hydroxide were prepared as required from A.R. pellets, a strong solution being initially formed in order to remove carbonate by precipitation. These solutions were standardised by titration against the standard hydrochloric acid, using methyl red as indicator. Potassium chloride solutions used in ionic strength adjustments were checked for neutrality against methyl red and thymol blue indicators.

*Solubility Determinations* (*Table I*)

Saturated solutions of piperazine adipate were obtained by stirring suspensions of the salt for 24-hour periods in vessels immersed in water baths thermostatically controlled to $\pm$ 0·2° C. of the desired temperatures. The suspensions were rapidly filtered through sintered glass funnels and the saturated filtrates titrated in suitable aliquots, after dilution with water, by the standard alkali, the titrations being followed potentiometrically.

*Determination of the Dissociation Exponents of Piperazine in Aqueous Solution at 25° C.* (*Table II*)

The determination was carried out upon the dihydrochloride, also used by Smith and Smith[13], but in the present instance the salt was not isolated. 25 ml. aliquots of a 0·1M solution of the hexahydrate were mixed with 50 ml. of 0·1N hydrochloric acid solution and diluted to 250 ml. by the addition of water. The acid solutions so obtained were titrated (25 ml. aliquots diluted to 50 ml. with water or standard potassium chloride solution) with 0·1N sodium hydroxide. The titrations were carried out in a jacketted cell, the temperature of which was maintained at 25° C. $\pm$ 0·2° C. The $pK'_a$ values so obtained were converted to thermodynamic values by application of the standard approximation of the Debye-Hückel relation, applicable within reasonable limits of error, where the ionic strength does not exceed 0·015.

## References


1. Welch, Peters, Bueding, Valk and Higashi, *Science*, 1947, **105**, 486.
2. Mouriquand, Roman and Coisnard, *J. med. Lyon*, 1951, **32**, 189.
3. Turpin, Cavier and Savaton-Pillet, *Therapie*, 1952, **7**, 108.
4. Standen and White, *Brit. med. J.*, 1953, **2**, 1272.
5. Gordonoff, *ibid.*, 1954, **1**, 394.
6. B.D.H. Ltd., Forrest and Petrow, B. Provisional Specification 29123/53; B.D.H. Ltd., Petrow and Sturgeon, B. Provisional Specification 15911/54; B.D.H. Ltd., Collins, Grenville, Petrow and Sturgeon, B. Provisional Specification 17454/54.
7. Jolly, H., *Private communication.*
8. Franklin, *Private communication.*
9. Hartley, *Brit. med. J.*, 1954, **1**, 521.
10. Sloan, Kingsbury and Jolly, *J. Pharm. Pharmacol.*, 1954, **6**, 718.
11. Kolthoff, *Biochem. Zeit.*, 1925, **162**, 289.
12. Bredig, *Z. phys. Chem.*, 1894, **13**, 313.
13. Smith and Smith, *Biol. Bull.*, 1949, **96**, 233.
14. Jeffery and Vogel, *J. chem. Soc.*, 1935, 21; Vogel, "*A Textbook of Quantitative Inorganic Analysis*," Longmans, Green & Co., 2nd Ed., 1951, p. 7.

# PIPERAZINE ADIPATE: A NEW ANTHELMINTIC AGENT

## PART II. TOXICOLOGICAL AND PHARMACOLOGICAL STUDIES

BY B. G. CROSS, A. DAVID and D. K. VALLANCE

*From the Biological Laboratories, The British Drug Houses Ltd., Godalming, Surrey*

Received July 7, 1954

THE purpose of this paper is to report the results of some pharmacological and toxicological investigations of piperazine adipate[1].

Pure piperazine adipate was used unless otherwise stated. In the acute toxicity studies in mice, pure and technical piperazine adipate and technical piperazine hydrate were examined simultaneously. To determine the effect on the hæmopoietic system of rabbits 250 mg./kg. was administered subcutaneously, but due to its low solubility[1] it was necessary to use the formulation as described in the text.

## RESULTS

### (*a*) *Acute Oral Toxicity*

#### (*i*) *Mice*

Male albino mice (ex-Evans, Stockwell), weighing approximately 20 g. each, were starved overnight and given one of the following preparations: pure piperazine adipate, technical piperazine adipate or technical piperazine hydrate. The compounds were administered as suspensions in 5 per cent. mucilage of acacia by means of a metal catheter, the 4 doses varying from 3 to 10 g./kg. The volumes were adjusted to 1 ml./20 g. of body weight. 10 animals were used at each dose, and from the mortalities after 7 days the LD50 and limits of error (P = 0·95) were calculated according to the method of Litchfield and Wilcoxon[2]. The results are recorded in Table I. All mortalities occurred within the first 24 hours, but there were

TABLE I

THE ACUTE ORAL TOXICITIES OF THREE PIPERAZINE COMPOUNDS IN MALE ALBINO MICE

| Compound | LD50 g./kg. | Limits of Error (P = 0·95) g./kg. |
|---|---|---|
| Piperazine Adipate (pure) .. | 11·4 | 9·2–14·0 |
| Piperazine Adipate (technical) .. | 8·2 | 7·0– 9·6 |
| Piperazine Hydrate (technical) .. | 4·3 | 3·3– 5·6 |

no immediate deaths. These results in mice demonstrate that on a weight for weight basis both the pure and technical piperazine adipate are considerably less toxic than technical piperazine hydrate.

#### (*ii*) *Rats*

Female albino rats (B.D.H. strain) weighing approximately 200 g. each were starved overnight, and piperazine adipate (10·0, 6·7, 4·5 and 3·0 g./kg.) was administered as a suspension in 5 per cent. mucilage of acacia by

means of a rubber catheter. The volume was adjusted to 5 ml./100 g. of body weight. 5 animals were used at each dose level and an additional group received an equivalent volume of the acacia alone.

Within 1 hour all the animals, including the controls, developed diarrhœa or passed fæces which although formed were softer than normal. The former condition was more evident in the groups receiving 10·0 and 6·7 g./kg. and persisted in some of those animals for 48 hours. No other toxic effects were observed apart from lethargy, which was very marked at the higher dose levels. There was no immediate mortaility, but at 10 g./kg. all the rats died within 24 hours and one rat given 6·7 g./kg. died on the second day following administration. No further deaths occurred during the 7-day observation period. The LD50 as estimated by Karber's[3] formula was 7·9 g./kg.

*(b) Subacute Oral Toxicity*

The possibility of cumulative toxic effects occurring on prolonged administration was studied by the addition of piperazine adipate to the diet (ground rat diet 41 as supplied by Associated London Flour Millers, Ltd.) of immature female albino rats (B.D.H. strain) for a period of 8 weeks. The amount of food was sufficient to satisfy hunger, and the piperazine adipate content was such that each animal received approximately 300 mg./kg. daily. 14 rats received this diet and 15, comprising the controls, received a diet identical in all respects other than the omission of the piperazine adipate.

During the experimental 8 weeks period each treated rat received a total of approximately 17 g. of piperazine adipate per kg. of body weight without the development of any apparent toxic effects. The animals were weighed at weekly intervals and no significant difference between the experimental and control groups was observed. The mean initial weight of the piperazine-treated rats was 116 g. (range 104 g. to 132 g.) and at the termination of the experiment the mean weight was 165 g. (range 141 g. to 185 g.). The mean initial and final weights of the control group were 114 g. (range 99 g. to 126 g.) and 162 g. (range 129 g. to 190 g.) respectively. The growth curves for the two groups are shown in Figure 1.

Autopsy of the animals at the end of the experiment revealed no gross pathological changes. Histological examination of lung, heart, liver, spleen, kidney, brain, spinal cord, peripheral nerve, stomach, small and large intestine did not reveal any significant difference between control and experimental groups.

*(c) Actions on Smooth Muscle*

The actions of piperazine adipate were studied on the isolated duodenum of rabbit and segments of guinea-pig's ileum suspended in a 50-ml. bath of ærated Ringer's solution at 35° C. The compound was added as a 4 per cent. aqueous solution. In the rabbit's duodenum preparations, no effects on the normal rhythmic contractions were observed with amounts below 10 mg. Larger amounts, from 10 to 40 mg., added to the bath for 2 to 8 minutes, caused an increase in tone (Fig. 2). This effect, however,

was reversed on washing out. Similar responses were obtained with piperazine citrate at the same dose levels. In the guinea-pig's ileum preparations, the spasmogenic action of piperazine adipate was again observed, with amounts varying between 4 mg. and 8 mg. The addition of 6 mg. of piperazine adipate produced a contraction which was 70 per cent. of the response caused by the addition of 0·2 $\mu$g. of acetylcholine. The response to piperazine adipate was, however, counteracted by the addition of methantheline bromide, a known spasmolytic, 0·3 $\mu$g. causing a 75 per cent. reduction in the response to 6 mg. of piperazine adipate.

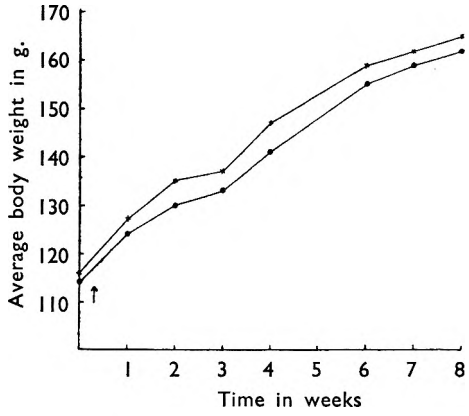


FIG. 1. Effect of piperazine adipate (300 mg./kg. per day) on the growth of immature female rats.
×——× Treated group.
●——● Control group.
↑ Indicates where treatment commenced.

The action of piperazine adipate on the intestine *in situ* was examined in 3 rabbits and 2 cats. The rabbits were anæsthetised with urethane (1 to 1·5 g./kg. intravenously) and the abdomens opened. The movements of a 3 cm. length of jejunum were recorded by attaching one end to a fixed rod and the other end to a small vertical lever whose movements were transmitted *via* a thread to a frontal writing lever. The abdominal cavity was

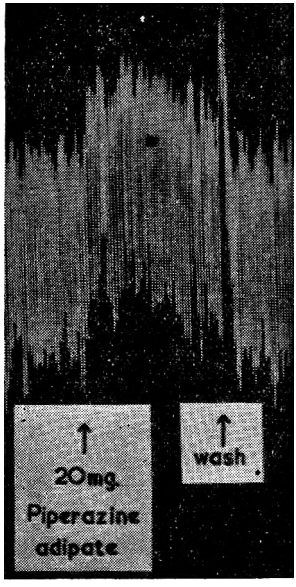


FIG. 2. Effect of piperazine adipate on the isolated rabbit duodenum (20 mg. in a 50-ml. bath for 4 minutes).

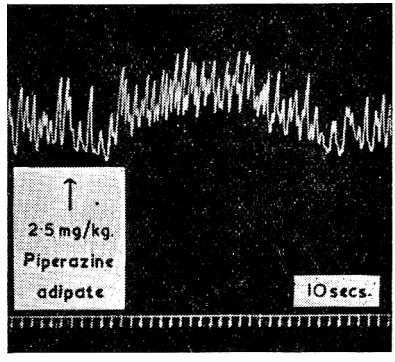


FIG. 3. Effect of intravenous injection of piperazine adipate (2·5 mg./kg.) on intact jejunum of a 3·8 kg. cat anæsthetised with chloralose.

filled with warmed Ringer's solution maintained at 37° C. Intravenous injection of varying amounts (3 to 15 mg./kg.) of piperazine adipate was generally followed by an increase in tone and a slight increase in the frequency of contractions. The cats were anæsthetised with ether followed by chloralose (50 to 60 mg./kg. intravenously). A water-filled balloon was placed in the jejunum through an abdominal incision, and the intestinal contractions were recorded by means of a water-air transmission system connected to a small piston recorder. Intravenous injections of piperazine adipate (1 to 2·5 mg./kg. in one animal and 5 to 20 mg./kg. in the other) were followed by an increase in tone, and occasionally by an increase in the frequency of contractions (Fig. 3).

*(d) Blood Pressure Responses in Anæsthetised Cats*

Blood pressure changes were recorded in 5 cats anæsthetised with ether followed by chloralose (60 to 80 mg./kg. intravenously[5]). Carotid arterial blood pressures were recorded by a mercury manometer, and intravenous injections of piperazine adipate given *via* the femoral vein. The amount of piperazine adipate required to produce a definite response varied considerably in the different animals. With one exception, however, amounts below 50 mg./kg. had no effect on blood pressure. Doses between 50 and 200 mg./kg. produced an immediate depressor response which rarely exceeded 50 mm. of mercury. 1 cat was particularly sensitive, a hypotensive response being elicited following 7 mg./kg. Recovery was rapid in all cases, the blood pressure returning to normal within 1 to 3 minutes following administration. The effect of atropine sulphate was examined in 1 animal; 0·6 mg./kg. failed to modify the depressor effect of piperazine adipate (60 mg./kg.).

The above responses occurred following the rapid intravenous injection of piperazine adipate. In contrast, the slow intravenous infusion of 500 mg./kg. over a period of 30 minutes had no demonstrable effect on blood pressure. The animal had, however, previously given typical depressor responses to 100 and 200 mg./kg. injected rapidly. The subsequent rapid injection of 100 mg./kg. again produced the typical response to rapid injection.

*(e) Effects on Heart*

*(i) In situ*

The action of piperazine adipate on the hearts of 2 cats, artificially respired under chloralose anæsthesia was studied *in situ*. A Cushny myocardiograph attached to the left ventricle was used to record the responses. Amounts of 50 to 120 mg./kg. in one preparation and 7 to 28 mg./kg. in the other, injected rapidly *via* the femoral vein, reduced the amplitude of the heart, but had only a slight effect on the rate (Fig. 4). The carotid blood pressure showed a concomitant fall. Recovery from both effects was rapid.

*(ii) Isolated Rabbit's Heart*

The cardiac effects were also studied on the isolated heart of the rabbit, perfused *via* the aorta through the coronary vessels with oxygenated

Ringer-Locke solution at 38° C. (Langendorff preparation). Piperazine adipate was administered by direct injections into the cannula. Records were obtained of the amplitude of the contractions and the rate was counted. Varying amounts (1 to 4 mg.) of piperazine adipate reduced the amplitude but only slightly reduced the rate (Fig. 5). Complete recovery occurred within 5 minutes. Smaller doses had little or no effect.

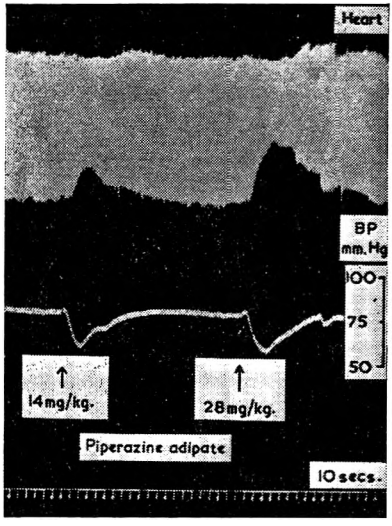


FIG. 4. Effect of intravenous injection of piperazine adipate (14 and 28 mg./kg.) on ventricular contractions and blood pressure in a 5·7 kg. cat anæsthetised with chloralose. Upper tracing: ventricular contractions; lower tracing: blood pressure.

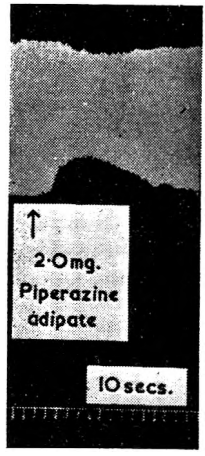


FIG. 5. Effect of piperazine adipate (2·0 mg.) on isolated perfused rabbit heart.

*(f) Effects on Respiration*

Respiratory effects were studied in 6 rabbits anæsthetised with urethane (1 to 1·5 g./kg.), using the method described by Gaddum[4]. The effects following the rapid intravenous injection of piperazine adipate were rather variable, but in general, doses varying from 2 to 16 mg./kg. produced slight stimulation of respiration, whereas larger doses had a depressant effect. The continued administration of piperazine adipate (4 per cent. solution) by fairly rapid intravenous injection resulted in death from respiratory failure in 2 rabbits. The doses required were 220 and 340 mg./kg. respectively.

*(g) Effects on Hæmopoietic System*

The effects of piperazine adipate on the hæmopoietic system were investigated on 8 female rabbits (B.D.H. stock) aged approximately 5 months. Due to solubility limitations the compound was given in the following preparation: piperazine adipate 10 g., phenol 0·2 g., sodium hydroxide solution B.P. 50 ml., distilled water 50 ml., and sufficient citric

acid B.P. to produce *p*H 7·4. Solutions so prepared were passed through a Seitz filter before use. A volume equivalent to 250 mg. of piperazine adipate per kg. of body weight was injected subcutaneously in 2 divided doses, 5 times a week for 5 weeks. A further 4 rabbits were given corresponding volumes of a formulation identical in all respects except for omission of the piperazine adipate.

Examination of weekly blood samples showed no significant differences between the red, white and differential cell counts or hæmoglobin concentrations of the experimental and control animals. The treated animals did not gain weight to quite the same extent as the controls. These results are summarised in Table II. By the 14th day of the experiment both groups developed slight ulceration at injection sites, and, at autopsy small hæmorrhages were observed in the underlying tissues. No significant gross or microscopical changes were evident in the major internal organs.

TABLE II

THE MEAN BODY WEIGHTS, RED, WHITE AND DIFFERENTIAL BLOOD-CELL COUNTS AND HÆMOGLOBIN VALUES OF 8 FEMALE RABBITS INJECTED SUBCUTANEOUSLY WITH THE EQUIVALENT OF 250 mg./kg. OF PIPERAZINE ADIPATE 5 TIMES A WEEK FOR 5 WEEKS AND OF 4 CONTROL ANIMALS GIVEN THE VEHICLE ALONE

| Group | Week | Weight, kg. | R.B.C. cells/ c.mm. | W.B.C. cells/ c.mm. | Hæmo-globin, per cent. | Lympho-cytes, per cent. | Mono-cytes, per cent. | Neutro-phils, per cent. | Eosino-phils, per cent. | Baso-phils, per cent. |
|---|---|---|---|---|---|---|---|---|---|---|
| Experimental 8 animals | Initial | 2·56 | 5,390,000 | 12,770 | 61 | 78·25 | 15·3 | 2·0 | 1·5 | 1·5 |
| | 1st week | 2·67 | 5,255,000 | 10,625 | 60·25 | 78·6 | 18·0 | 2·0 | 0·25 | 1 |
| | 2nd ,, | 2·66 | 5,522,000 | 10,870 | 60·6 | 74·2 | 21·5 | 2·0 | 1 | 1·5 |
| | 3rd ,, | 2·66 | 5,430,000 | 10,900 | 60·0 | 75·5 | 21·5 | 1·5 | 1 | 0·25 |
| | 4th ,, | 2·66 | 5,500,000 | 10,100 | 59·5 | 72·75 | 24·0 | 2·0 | 0·6 | 0·5 |
| | 5th ,, | 2·68 | 5,329,000 | 10,900 | 60 | 74·5 | 21·3 | 2·0 | 2 | 0·25 |
| Control 4 animals | Initial | 2·52 | 5,420,000 | 12,300 | 61·7 | 79·0 | 16·0 | 3 | 0·75 | 1 |
| | 1st week | 2·71 | 5,500,000 | 12,100 | 62·0 | 80·5 | 16·5 | 2 | 0·25 | 0·75 |
| | 2nd ,, | 2·7 | 5,670,000 | 12,350 | 62·0 | 79·5 | 17·0 | 1·75 | 0·75 | 1 |
| | 3rd ,, | 2·87 | 5,650,000 | 11,650 | 61·5 | 74·5 | 20·25 | 1·25 | 1·75 | 0·25 |
| | 4th ,, | 2·84 | 6,000,000 | 11,500 | 62·0 | 75·0 | 18·25 | 2 | 1·25 | 1·0 |
| | 5th ,, | 2·9 | 5,480,000 | 12,100 | 61·25 | 77·5 | 18·0 | 2·25 | 1·25 | 0·75 |

## DISCUSSION

This investigation has shown that piperazine adipate is comparatively non-toxic, the oral LD50 of the pure compound in mice and rats being 11·4 and 7·9 g./kg. respectively. This provides an ample margin of safety as the maximum dose recommended for clinical use would not normally exceed 75 mg./kg. a day for 7 days. The only toxic effects observed in rats following single sublethal doses were lethargy and diarrhœa. The spasmogenic effect obtained on isolated intestinal muscle and intestinal muscle *in situ* might possibly be of significance in the latter connection, although the amounts required to produce such responses were undoubtedly very large.

It is evident that therapeutic oral doses in man are unlikely to cause either circulatory or respiratory disturbances. These effects were only observed following the rapid intravenous injections of extremely large doses.

Finally, the continued administration of 300 mg./kg. daily in the diet of immature rats for 8 weeks and the subcutaneous injection of 250 mg./kg.

daily (5 times a week) for 5 weeks in rabbits did not produce any pathological changes, apart from local necrosis at injection sites, probably attributable to the concentrated preparation used. In particular piperazine adipate did not have any deleterious action on the hæmopoietic system of rabbits.

## Summary

1. An investigation on animals of the toxicological and pharmacological properties of piperazine adipate, a new anthelmintic agent, is reported.

2. It is comparatively non-toxic orally, the LD50 of mice and rats being 11·4 and 7·9 g./kg. respectively.

3. It has a spasmogenic action on isolated intestinal muscle and intestinal muscle *in situ* when large doses are given.

4. Cardiac, circulatory and respiratory effects were slight, the rapid intravenous injection of large amounts being required to produce a response.

5. It has been safely administered over a prolonged period to rats and rabbits.

The authors thank Dr. S. W. F. Underhill for his valuable advice and the Directors of The British Drug Houses, Ltd., for permission to publish these results.

## References

1. Davies, Forrest, Hartley and Petrow, *J. Pharm. Pharmacol.*, 1954, **6**, 707.
2. Litchfield and Wilcoxon, *J. Pharmacol.*, 1949, **95**, 99.
3. Karber, *Arch. exp. Path. Pharmak.*, 1931, **162**, 480.
4. Gaddum, *J. Physiol.*, 1941, **99**, 257.

# PRELIMINARY TRIALS WITH PIPERAZINE ADIPATE AS A VETERINARY ANTHELMINTIC

By J. E. N. Sloan, P. A. Kingsbury and D. W. Jolly

*From the Cooper Technical Bureau, Berkhamsted*

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## Introduction

During the last few years we have examined some 40 piperazine compounds for evidence of anthelmintic activity against the nematodes commonly found in domestic animals. Our trials were begun following the publication of the good results obtained when diethylcarbamazine acid citrate (hetrazan) was used to treat filariasis (Hewitt *et al.*[1]), and to control ascarids in dogs and cats (Hewitt *et al.*[2] and Kanegis[3]). The high cost of hetrazan makes it impracticable for treatment of farm animals and attention was directed to the anthelmintic properties of cheaper piperazine derivatives.

A high level of toxicity or lack of therapeutic effect rendered the majority of compounds tested quite unsuitable for practical use. Piperazine hexahydrate, recently shown to be of value for treatment of Enterobius in man (White and Standen[4]) was found to be an effective ascarifuge in dogs and cats at an early stage in our trials. However, the physical nature and nauseating taste of this compound seriously detracted from its value for treatment of these animals. At this stage we were invited to evaluate the veterinary applications of piperazine adipate[5] which has none of the disadvantages associated with the other compounds tested and it was rapidly established that besides being effective in cases of Enterobius in man, it showed very useful properties as a veterinary anthelmintic. The initial trials made against the ascarids of dogs were so successful that the work was extended to include tests against ascarids in horses, pigs and poultry. This paper briefly records the good results obtained against these and other species of nematodes in the various hosts.

## Materials and Methods

Pure piperazine adipate was used in these experiments. 3 horses were drenched with a suspension of the drug in water and 2 ate it mixed in bran mash. On 13 other occasions the drug was administered to these animals by stomach tube. It was given to pigs mixed in wet mash and to poultry in dry mash and occasionally in wet mash or in capsules. The majority of the dogs and cats were treated with piperazine adipate in gelatine capsules, the remainder received it in the form of tablets or ate it as powder mixed with their food.

No preliminary fastings, except in 4 dogs, nor post-dosing treatment with purgatives, etc., was carried out. Normal feeding procedure was followed and water was always freely available.

Evaluation of anthelmintic efficiency required different procedures according to the host species. A direct critical test—worms expelled/worms

remaining—was possible in all cases with poultry as these could be slaughtered. A similar critical figure was obtained for 6 dogs. The remaining dogs and the cats could not be sacrificed, but all worms expelled by treatment were collected and comparisons made between pre-dosing and post-dosing fæcal egg counts. A similar procedure was used for the horses and pigs. Where possible individual dogs, pigs, and horses received 2 or 3 successively larger doses at weekly intervals, the final dose being co-ordinated with the toxicity observations. By this means worms not removed by the small dose at the first treatment were expelled by a much bigger one the week after and a maximum percentage efficiency figure for the initial small dose could be established.

The toxicity of piperazine adipate has been investigated by Cross *et al.*,[6] who found the anthelmintic to be practically non-toxic. Our toxicological observations were therefore designed to ascertain if a toxic response could be elicited by the oral administration of as much of the anthelmintic as its bulk would allow, using the conventional methods of dosing which obtain in practice. These amounts were from 3 to 5 times the therapeutic dose. All the animals under test were examined clinically, their food intake and behaviour observed, and blood tests completed. In cases where animals were sacrificed, a post-mortem examination was made, and sections of liver and kidney were microscopically examined.

## Experimental Results

### 1. Dogs

*Ascarids. Toxocara canis* and *Toxascaris leonina.*

13 dogs, mostly infected with *T. leonina*, were dosed once with piperazine adipate at rates varying from 25 to 200 mg./kg. of body weight. 193 ascarids were expelled after treatment and subsequent fæcal examinations over a period of several weeks revealed no ascarid eggs. A post-mortem examination was made on one dog which had expelled ascarids after dosing, no further worms remained. An anthelmintic efficiency of 100 per cent. is indicated.

*Hookworms. Ancylostoma caninum* and *Uncinaria stenocephala.*

16 dogs infested with *A. caninum* were dosed at rates ranging from 100 mg./kg. to 300 mg./kg. on 3 successive days. 372 hookworms were removed by the dosing, but in no case did the fæces become negative for hookworm eggs even after repeated treatments. Post-mortem examination of 4 dogs showed that dosing had removed only 22/81 worms. Taking all our data together it is concluded that an average efficiency of more than 40 per cent. cannot be obtained against this species by the use of single doses up to 300 mg./kg. of body weight. The northern hookworm, *U. stenocephala*, which is a less sturdy parasite than *A. caninum*, appears to be more susceptible to piperazine adipate. 2 dogs were given 100 mg./kg. of body weight and expelled 38 worms. A post-mortem examination showed that all the worms present in both dogs had been removed by the treatment, indicating an efficiency of 100 per cent. 5 more dogs infected with

*Uncinaria* were treated with one dose of 100 mg./kg. Hookworm eggs completely disappeared from the fæces of 4 of them and in the fifth, which is known to have expelled at least 94 worms, the fæcal egg count fell from 2000 to 150 eggs per g.

*Cestodes. Tænia hydatigena* and *Diplylidium canium.*

9 of the dogs were infected with *T. hydatigena* and 2 with *D. caninum* in addition to their nematode infestations. Piperazine adipate showed no apparent anthelmintic effect against these common tapeworms.

*Toxicity*

2 dogs, 18 months old, and weighing 12 and 15 kg., received a total of 26 and 27 g. of piperazine adipate over a period of 18 weeks, which represented an intake of 1800 and 2170 mg./kg. This dosing routine was finished by the administration of 6 and 9 g. of piperazine adipate in 72 hours, which is equivalent to 500 mg./kg. The fæces of both dogs were softer than normal, although the shape was maintained. No ill effects were detected, except that one observer, who knew both dogs well, was of the opinion that there was some evidence of slight hypersensitivity for 24 to 48 hours after dosing with 500 mg./kg. of piperazine adipate.

2. FELINES

*Ascarids. Toxocara mystax* and *Toxascaris leonina.*

3 cats infected with ascarids were dosed at the rate of 100 mg./kg. of body weight. All the post-dosing fæcal material could not be collected, but it is known that many worms were expelled. Subsequent fæcal examinations remained negative and a 100 per cent. efficiency seems probable.

One lion and 4 lionesses infected with *T. leonina* were dosed with *ca.* 100 mg./kg. of body weight, of the drug given concealed in gutted rabbits which were readily eaten. At least 496 worms were expelled by the treatment and probably more which could not be collected. Subsequent fæcal examinations indicated that 3 of the beasts were cured and that the infestations in the other 2 were markedly reduced.

*Palatability.*

4 dogs and 1 cat ate doses of the drug equivalent to 100 mg./kg. of body weight quite readily in their ordinary meat or fish food. They did not vomit afterwards and throughout the dosing trials too, there was a complete absence of this undesirable feature which so commonly accompanies anthelmintic treatment in these species.

3. PIGS

*Ascaris lumbricoides.*

7 lightly infected store pigs were treated with doses of 150 to 600 mg./kg. of body weight in wet mash. The lower dose rate was ineffective, but higher rates led to removal of 31 worms and subsequent fæcal examina-

tions remained negative for ascaris eggs. A dose rate of 300 to 400 mg./kg. to a maximum of 40 g. seems to be necessary for a 100 per cent. cure.

*Œsophagostomum spp.*

21 pigs carrying light to moderate infestations of nodular worms were dosed at an estimated rate of 300 to 400 mg./kg. of body weight. 1010 worms were expelled from 1 group of 12 lightly infested animals. Only 2 of these ceased to pass eggs in the fæces after treatment, the remainder retaining small residual infestations. Complete post-dosing samples could not be obtained from the 9 other pigs, but 1025 worms were recovered from the relatively small quantity available and the total number passed must have reached several thousands. Comparison of mean pre- and post-dosing fæcal worm-egg counts showed a drop from 1100 to less than 50 eggs per g. Efficiency against these worms is probably 80 to 90 per cent. at practicable dose rates.

*Toxicity.*

A group of 6 pigs, 4 to 6 months old, received 450 g. of piperazine adipate daily in their food for 3 days, which represented a total individual intake of 1875 mg./kg. The medicated food was taken readily on the first day, but eaten reluctantly on the second and third days. No abnormality, apart from a loss in weight which approximated 2 pounds per pig during the period of the test, was observed. A normal weight gain was observed the week following the test.

4. EQUINES

6 yearling Dartmoor ponies were used in the trials. Their weights were approximately 100 to 120 kg. Initially they carried light infestations of ascarids, small redworms and pinworms and, after being given low-level doses of piperazine adipate to indicate if it had any possible potentialities in this host, their ascaris and pinworm burden was increased by administrations of infective eggs. When the infections had had time to proceed towards maturity the animals were dosed at the 250 to 400 mg./kg. of body weight level followed by 1250 to 1500 mg./kg. a week later. The very small number of worms removed by the big final doses and the fall to zero of the ascarid and strongyle fæcal egg counts in all the 6 ponies at the end of the tests left little doubt that the doses of 250 to 400 mg./kg. given the previous week had been highly effective and already removed the great majority of parasites originally present.

*Toxicity.*

Some increase in the liquid content of the fæces was observed for 24 hours when the ponies received 1250 to 1500 mg./kg. of piperazine adipate administered in 5000 ml. of water by stomach tube. No other abnormality was detected.

*Ascarids. Parascaris equorum.*

Dose rates of 120 to 150 mg./kg. of body weight were ineffective, 250 mg./kg. apparently removed 14/16 worms and 400 mg./kg. apparently removed all of 51 worms, ranging from small 100 mm. stages to mature

adults, from 3 horses. Efficiency against equine ascarids was further confirmed when 2 young zebras given *ca.* 300 mg./kg. of body weight of piperazine adipate in their food expelled at least 75 adult ascarids and over 100 immature ones.

*Pinworms. Oxyuris equi.*

The anthelmintic effect against the adult females was similar to that against ascarids. 250 mg./kg., removed about 80 per cent. of some 800 of them and there was an apparent 100 per cent. cure in two horses at 400 mg./kg.—211 worms being removed and no more appearing after the subsequent 1250 to 1500 mg./kg. dose a week later. The males and larval stages proved more resistant—only 60 to 90 per cent. appeared to be removed at the 250 to 400 mg./kg. dose rates. 3636 were expelled altogether by the 18 dosings given.

*Small strongyles.* Mainly *Trichonema spp.*, and *Triodontophorus spp.*

Doses below 250 mg./kg. of body weight were not very effective, but judging by the numbers expelled, 11,221 in all, and the drop in fæcal egg counts, a minimum efficiency of 85 per cent. can be expected at a 400 mg./kg. dose rate in ponies of this size. A rather lower dose rate would probably be satisfactory in bigger horses in which the gut volume would be relatively smaller.

5. POULTRY

*Ascarids. Ascaridia galli.*

109 infected 14-week-old cockerels were available. 70 were treated with piperazine adipate and 39 kept as controls. All worms expelled were collected and each bird was then examined post-mortem, and the remaining worms counted. These trials are summarised in Table I. 1537 worms were expelled by treatment and 47 left, giving an anthelmintic efficiency of 97 per cent. When more than 150 mg./kg. of body weight was given or taken by the birds in wet or dry mash the efficiency was always greater than 96 per cent. (62 birds treated). After individual dosing *Ascaridia* began to appear in the fæces at 3 hours, and were nearly all expelled by 6 hours. After administration in the food the majority were expelled within 24 hours of commencing the trial. An additonal test in 12 laying hens showed that treatment caused no fall in egg production compared with 12 control birds.

*Toxicity.*

Groups of 12-week-old birds were dosed with piperazine adipate powder in capsules at the rate of 1000 mg./kg. and 2000 mg./kg. The lower rate is 5 times and the higher rate 10 times the therapeutic dose. No ill effects were observed at the lower dosage level. At the higher level the birds were lethargic and disinclined to feed for 1 to 3 hours after dosing, but had completely recovered by the fifth hour. Some scouring was apparent for a few hours, but the fæces became normal again within 24 hours. Other birds were fed for 24 hours on dry mash containing piperazine adipate

powder at the rate of 1 part powder to 35 parts mash, which gives 10 times the therapeutic dose. At this concentration the medicated mash proved slightly unpalatable, but the birds achieved an intake of 4 g. of powder per head in the 24 hours. All the birds remained entirely normal.

TABLE I

DETAILS OF POULTRY EXPERIMENTS

| Number of birds dosed | Number of control birds | Mode of administration of drug | Days fed | Amount of drug taken by each bird in one day, mg./kg. | Worms removed / Worms present pre-dosing | Worms removed by dosing, per cent. | Worms expelled naturally by controls, per cent. |
|---|---|---|---|---|---|---|---|
| 4 | 4 | Single dose in capsule | | 100 | 95/130 | 73 | 0 |
| 4 | 4 | " " " " | | 150 | 107/110 | 97 | 0 |
| 4 | 4 | " " " " | | 300 | 58/58 | 100 | 0 |
| 6 | 5 | " " " " | | 440 | 69/69 | 100 | 0 |
| 10 | 4 | " " " " or one 300 mg. tablet per bird | | ca 400 | 248/248 | 100 | 0 |
| 4 | 0 | 1 part powder to 700 parts dry mash fed *ad. lib.* | 3 | 138 | 67/71 | 94 | – |
| 6 | 6 | 1 part powder to 500 parts dry mash fed *ad. lib.* | 3 | 202 | 103/103 | 100 | 7 |
| 8 | 4 | 1 part powder to 500 parts dry mash fed *ad. lib.* | 3 | 220 | 174/175 | 99 | 5 |
| 12 | 0 | 1 part powder to 500 parts dry mash fed *ad. lib.* | 3 | 264 | 440/442 | 99·5 | – |
| 4 | 4 | 1 part powder to 350 parts dry mash fed *ad. lib.* | 2 | 297 | 29/29 | 100 | 0 |
| 4 | 4 | 1 part powder to 350 parts dry mash fed *ad. lib.* | 1 | 330 | 72/72 | 100 | 0 |
| 4 | 0 | 1 part powder to 350 parts wet mash in two feeds | 1 | 250 | 75/78 | 96 | – |

## DISCUSSION

The preliminary trials with piperazine adipate which are summarised above indicate that this compound shows promise of proving a highly efficient ascaricide with such a wide margin of safety that it will be of considerable value in veterinary therapeutics. The facility with which it can be administered in both wet and dry food, the apparent lack of irritant effects on the gut, mucosa and other toxic manifestations, the absence of any need for supporting therapy such as purgatives, and an anthelmintic activity which is by no means restricted to members of the *Ascaroidea*, give this drug many advantages over those in current use.

The anthelmintic properties and lack of toxicity suggest that it would provide a valuable method of treatment for ascaris, redworm and oxyurid infections in horses, especially young thoroughbreds, which suffer severely from ascarids. In pigs, efficiency against ascarids and nodular worms would eliminate the necessity of using sodium fluoride for the former and phenothiazine for the latter and the possibility of giving the drug in wet food would obviate the risk of toxicity that is run when sodium fluoride is given in this way. The absence of vomiting after dosing is a valuable asset in treatment of dogs and cats for ascarids and the possibilities for administration in the food render it suitable for treatment of similar worm species frequently found in exotic carnivores. Further trials in progress

may show that it will also give useful control of at least some species of hookworms in those hosts in which use of carbon tetrachloride and similar compounds is not always possible or even advisable.

Virtually 100 per cent. control of *Ascaridia* is possible in poultry and piperazine adipate has advantages over carbon tetrachloride treatment in respect of likely toxicity and over phenothiazine-nicotine-bentonite in that it can be given in wet as well as dry mash and there is no subsequent feather staining by the excretory products derived from phenothiazine.

The authors thank the British Drug Houses, Limited, for the piperazine adipate used in these trials and acknowledge with thanks the help given to them in some of these trials by Mrs. M. Leadbetter, of Southborough, Kent; by the veterinary staff of the Greyhound Racing Association Kennels, Potters Bar, Middlesex, by the Animal Health Trust Equine Research Station, Newmarket, and by Mr. E. H. Tong, Superintendent, and Mr. M. Senior, M.R.C.V.S., at Whipsnade Zoological Park, Beds. They are also indebted to Mr. W. Downing, F.R.C.V.S., for assistance in the trials with horses and to Messrs. Cooper, McDougall and Robertson, Limited, Berkhamsted, for permission to publish this paper.

## References

1. Hewitt *et al.*, *J. Lab. clin. Med.*, 1947, **32**, 1314.
2. Hewitt *et al.*, *J. Parasitol.*, 1948, **34**, 237.
3. Kanegis, *J. Amer. vet. med. Assoc.*, 1948, **113**, 579.
4. White and Standen, *Brit. med. J.*, 1953, **2**, 755.
5. Davies, Forrest, Hartley and Petrow, *J. Pharm. Pharmacol.*, 1954, **6**, 707.
6. Cross, David and Vallance, *ibid.*, 1954, **6**, 711.

# ON THE MAINTENANCE OF STERILITY IN EYE-DROPS

By M. Klein, E. G. Millwood and W. W. Walther
*From Whipps Cross Hospital, Leytonstone, London, E.*11

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The use of preservatives in eye drops and ophthalmic solutions is not new, and *p*-hydroxybenzoic esters have been advocated to prevent contamination (for review of earlier references see Klein[1]), replacing chlorocresol (0·03 per cent.), which was included in the earlier editions of the National Formulary. The preservatives were added to eye solutions mainly to prevent the growth of moulds, and laboratory tests were carried out mostly with this object in view.

Recently, however, severe eye infections have been caused with eyedrops contaminated with *Pseudomanas pyocyanea* (*Ps. aeruginosa*). McCullogh[2] reported 18 cases of pyocyanea infection, 5 of which could be traced to contaminated eye drops. On testing the eye drops McCullogh found that almost any commonly used drops could become contaminated with *Ps. pyocyanea*, but fluorescein and eserine bottles collected from the hospital wards were almost always contaminated. In Bignell's[3] series of severe pyocyanea keratitis, most of the cases were due to contaminated penicillin solutions instilled into the eye after superficial injuries. Other authors traced the source of pyocyanea infection to distilled water used in the theatre after an operation, or to the water used to make up solutions, and several cases were reported by different speakers at the meeting of the Ophthalmological Society of the United Kingdom in 1953. Rintelen[4] reported 4 cases of fulminating endophthalmitis due to contaminated tannic silver proteinate solution used after cataract operations. *Escherichia coli* and nonhæmolytic streptococci were isolated from the distilled water used in the preparation of the solution. This was surprising because solutions of silver salts are usually regarded as being bactericidal. Several cases of pyocyanea contamination of cortisone have occurred in this country, and Theodore[5] gave instances of commercial drops having to be withdrawn from sale because of pyocyanea infection.

Soet in 1952 (quoted by King[6]) reported the loss of eyes of several workers in a factory, through pyocyanea infection caused by contaminated eye-drops used in first-aid posts.

Theodore and Feinstein[7] drew attention to the danger of contamination where hospital pharmacists prepare large stocks, and recommend a careful method for the preparation and handling of the solutions.

Other contaminations have also been found, such as *Proteus* in methylcellulose, and Thygesson in 1949 (quoted by Theodore) reported virus infections which were transmitted by eye-drops.

The National Formulary generally follows the line of the British Pharmaceutical Codex regarding the use of bacteriostatic agents, using Liquor pro Guttis B.P.C., as the general solvent for eye-drops, the formula of this being 0·023 per cent. of methyl hydroxybenzoate and 0·011 per cent.

of propyl hydroxybenzoate in freshly boiled and cooled distilled water. It is interesting to observe that the official fluorescein eye-drops are not required to contain any bacteriostatic or germicidal agent whatsoever. The vehicle consists simply of a solution of sodium chloride in sterilised distilled water. The eye lotions in the National Formulary contain no preservative.

The British Pharmacopœia, the British Pharmaceutical Codex and National Formulary require that aseptic precautions shall be observed in the manufacture and dispensing of ophthalmic preparations. Although sterility in any preparation used for treatment is essential, it is difficult to maintain and the dispensing of sterile solutions is not a sufficient safeguard, as the possibility of contamination from aerial sources or from direct contact exists as soon as a bottle is opened. If the eye-drops used in a factory first-aid post or treatment room, or an outpatient department, become contaminated the consequences are especially grave, and several patients may be affected before the trouble is located. There should be adequate safeguards to ensure the continued sterility of all solutions used in the diagnosis and treatment of eye conditions.

To keep solutions free from contamination different methods have been suggested, Morris and Truhlsen[8], and others advocated heat sterilisation for drops used in the theatre before and after operations. The potency of the drugs can, however, be affected by heating, and the solution may soon become contaminated once the bottle has been opened. For this reason Haffley and Jensen[9] suggested rubber-capped vaccine vials, the solutions being drawn up by means of a sterilised syringe. This method, however, has limited use. Chemical preservatives such as chlorocresol, chlorbutol and *p*-hydroxybenzoates have been used, but their effectiveness against *Ps. pyocyanea* needs to be verified. Quaternary ammonium compounds were recommended by Hughson and Styron[10], Kedvessy, De Grosz and Szepes,[11] Macpherson and Wood[12] and others. Mostly benzalkonium chloride was used in concentrations varying from 1 in 5000 to 1 in 20,000. Mercurial preparations thiomersalate 1 in 20,000 and metaphen 1 in 7,000 were tried by McCullogh[2].

## Comparative Efficiency of Various Antiseptics and Preservatives

In the present work we tested the ability of a number of substances to maintain the sterility of a solution against infection by *Ps. pyocyanea* (*Ps. aeruginosa*). 3 strains were used—N.C.T.C. 7244, N.C.T.C. 5083, and one isolated from a human source that had been used to produce experimental corneal ulcers in rabbits (Klein and Millwood[13]). Testing for bactericidal effect in distilled water was found to be impracticable because no visible difference existed in tubes containing viable organisms compared with tubes in which the organisms had been killed by the test substance. Therefore it was decided to make up the solutions in a simple nutrient medium, and Needham's broth was chosen, the infection being carried out under standardised conditions. A range of dilutions of each substance was made in order to discover the concentration required to prevent infection of the solution when inoculated with 0·02 ml. of a culture of

pyocyanea, maintained as suggested by Needham[14] and containing approximately $14 \times 10^9$ viable bacteria per ml. After inoculation the solutions were incubated at 37° C. for 48 hours, and then 0·1 ml. was sub-cultured from the tubes showing no obvious growth into 20 ml. of Needham's broth and a similar quantity into a tube of thioglycollate

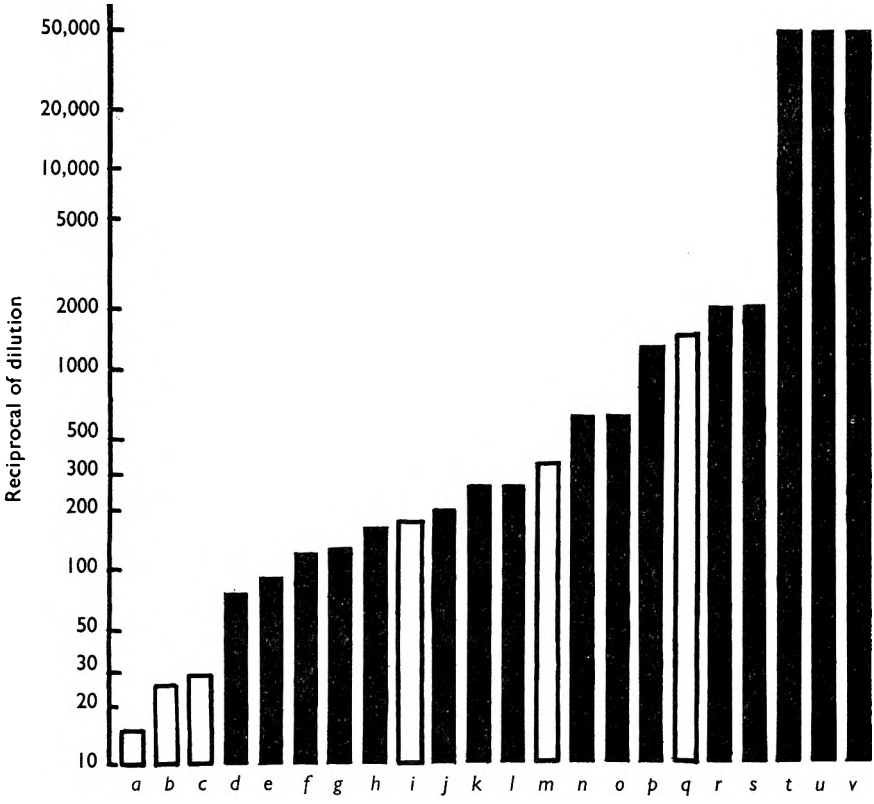


FIG. 1. Relative efficiency of antiseptics in Needham's broth, incubated at 37°C. for 48 hours.

(*a*) methanol; (*b*) ethanol; (*c*) urethane; (*d*) β-phenoxyethylalcohol; (*e*) β-phenoxypropyl-alcohol; (*f*) *iso*octylhydrocupreinotoxin; (*g*) benzyl alcohol; (*h*) chlorobutol (dissolved without heat); (*i*) lysol B.P.; (*j*) *p*-chlorophenyl-α-glycerol ether; (*k*) β-phenylethyl alcohol; (*l*) methylphenyl carbinol; (*m*) phenol; (*n*) hydroxybenzoic acid ester; (*o*) hydroxybenzoic acid ester (Nipa 82121); (*p*) chlorocresol; (*q*) sodium azide; (*r*) benzalkonium chloride; (*s*) cetrimide; (*t*) phenylmercuric acetate; (*u*) phenylmercuric nitrate; (*v*) thiomersalate.

medium, these then being incubated for a further 48 hours. The results obtained from the 3 strains of pyocyanea did not vary greatly and the dilution of the substance which maintained sterility against the least sensitive strain was recorded.

In Figure 1, which shows the relative efficiency of substances, the white columns represent compounds used for comparison only. They are methanol, ethanol, urethane, lysol B.P., phenol, and sodium azide. It may be seen that this test is a strict one, since the organism was placed in a

medium favourable to its growth, and if the same substances were used for the preservation of eye-drops a lower concentration should suffice. Phenoxyethyl alcohol, and phenoxypropyl alcohol were effective in 1 in 80 dilution. *iso*Octylhydrocupreinotoxin which has been recommended as a preservative for injection solutions, and which kills staphylococci and streptococci in over 1 : 100,000 dilution, was only moderately effective, and in the effective concentration the solution was cloudy and its use is not recommended. Benzyl alcohol 0·9 per cent. is used as a preservative in the original stock solution of cortisone acetate. In that concentration it proved effective against pyocyanea. When, however, cortisone eye-drops are made up, the cortisone is diluted in the proportion of 1 : 4, and the concentration of benzyl alcohol falls below the effective level. This would explain the pyocyanea contamination of cortisone eye-drops. It seems rational therefore to use 0·9 per cent. of benzyl alcohol in distilled water or saline solution in making up cortisone eye-drops.

Chlorbutol enjoyed widespread use as a preservative for some time. In many tests our results were inconsistent and on looking up previous publications we found that the recommended concentration varied from saturated solutions (approximately 0·8 per cent.) to 0·3 per cent., and even lower, and by some it was regarded as unreliable. This erratic behaviour of chlorbutol may be explained by the fact that it is a volatile substance and if a solution is made up by heating, or the water preserved with chlorbutol is boiled before making the eye-drops, the chlorbutol may escape or be decomposed by heating. The practical effect of this is shown in Table I.

*p*-Chlorphenyl-α-glyceryl ether (Gecophen) was effective in 0·5 per cent. solution. Phenylethyl alcohol and its isomer methylphenyl carbinol were both effective in 0·4 per cent. solution. Our findings agree with those of Brewer, Goldstein and McLaughlin[15]. The *p*-hydroxybenzoates are recommended in the National Formulary as inhibiting and not as bactericidal agents. A combination of these esters (Nipasept) was used, and against pyocyanea it was effective at 0·16 per cent., which is the limit of its solubility and is several times the strength recommended by the N.F. A new combination of *p*-hydroxybenzoate (Nipa 82121) was supplied by the makers, and in Needham's broth its effectivity was the same, but when used in eye-drops it proved more potent against pyocyanea than the commercially available "Nipasept." Chlorocresol is perhaps the most time-honoured preservative for eye-drops. It was found that in Needham's medium it was efficient at less than 0·1 per cent. In this concentration it killed a heavy contamination of pyocyanea in a very short time.

Among the quaternary ammonium compounds benzalkonium chloride was effective in 1 in 2000 and several commercial preparations gave comparable readings. Cetrimide was effective against pyocyanea in 1 : 2000, but one batch had a very moderate bactericidal effect even at a concentration of less than 1 in 100. Quaternary ammonium compounds as a preservative for eye-drops should be used only in exceptional cases. Ginsburg and Robson[16] found that detergents could prove harmful by causing "solubilisation" of the intercellular cement of the corneal

epithelium. For the sterilisation of surgical instruments or acrylic implants its use is permissible if followed by a thorough rinse in distilled water or saline solution.

Of the mercurial group phenylmercuric nitrate, phenylmercuric acetate and thiomersalate were tested, and all of them found to be effective in high dilutions. Their use has been recommended for eye-drops and also for solutions for injections. They are not only potent bactericides, but are also effective fungicides.

## Tests on Eye-drops

The tests previously described were made in Needham's medium, but from a practical point of view it seemed desirable to test some of these substances on eye-drops as used in everyday practice. For the tests we selected 0·5 per cent. atropine sulphate, 0·25 per cent. eserine salicylate and 2 per cent. sodium fluorescein dissolved in distilled water. The last two are readily contaminated with pyocyanea. From the many substances in the comparative efficiency diagram we selected arbitrarily a few only and serial tests were made with them. The eye-drops were prepared with preservative and then infected with 0·02 ml. of an 18-hour culture of *Ps. pyocyanea*. Subcultures were then taken at intervals up to 24 hours. The results are recorded in Table I.

Chlorocresol (Table I) was tested in 0·1 per cent. and 0·03 per cent. concentration. The 0·1 per cent. kills *Ps. pyocyanea* almost instantaneously and in spite of the massive infection it remains sterile. With the 0·03 per cent. solution atropine became sterile in between 2 and 4 hours, while fluorescein and eserine needed more than 6 hours.

Chlorbutol (Table I) 0·5 per cent. was used and the difference between the heated and unheated solutions is significant. It seems that chlorbutol if used as a preservative for eye-drops must be dissolved without heat. Theodore's suggestion that undissolved chlorbutol crystals should be present in the dropper bottle, and when the crystals have dissolved, that new ones should be placed in the bottle with sterilised forceps, is a safeguard, otherwise, since chlorbutol is so volatile its concentration in solution may be dropping below the effective level.

Phenylethyl alcohol was recommended in 0·5 per cent. solution, but the tests (Table I) show that 0·6 per cent. is safer, killing pyocyanea within 1 hour. With the 0·5 per cent. phenylethyl alcohol, pyocyanea was viable for more than 3 hours. When the drops are used in an outpatient department this difference may be of importance.

Thiomersalate (Table I) 0·005 per cent. solution worked best with fluorescein, where it killed pyocyanea in less than 2 hours, while in atropine it needed over 5 hours.

The *p*-hydroxybenzoates—Combination 1 ("Nipasept") and Combination 2 (Nipa 82121) were tested in 3 different strengths. The tabulated results (Table I) show that in fluorescein their antipyocyanea effect is not satisfactory. In atropine and eserine, the new combination is better than the old one.

In further tests we used thiomersalate 0·002 per cent. and chlorocresol

0·1 per cent. with 0·5 per cent. atropine sulphate. 2 bottles of each solution were prepared, one being kept at room temperature and the other at 37° C. to see if temperature affected the efficiency of the preservative. For a period of 31 days the bottles were opened at intervals of 2 to 3 days and the contents tested for sterility, and one drop of an 18-hour culture of *Ps. pyocyanea* containing approximately $14 \times 10^9$ viable bacteria then added. By the end of the test period the bottles had been opened and

TABLE I

| Eyedrops | Preservative, per cent. | Hours after infection with 0·02 mL of 18-hour culture of *Ps pyocyanea*. + indicates growth when subcultured. 0 | 1 | 2 | 3 | 4 | 5 | 6 | 21 to 24 |
|---|---|---|---|---|---|---|---|---|---|
| Sodium Fluorescein 2 per cent. | Chlorocresol 0·1 | − | − | − | − | − | − | − | − |
| | Chlorocresol 0·03 | + | + | + | + | + | + | + | − |
| | Chlorbutol 0·5 (dissolved without heat) | + | + | − | − | − | − | − | − |
| | Chlorbutol 0·5 (dissolved with heat) | + | + | + | + | + | + | + | − |
| | Thiomersalate 0·005 | + | + | − | − | − | − | − | − |
| | β-Phenylethyl alcohol 0·5 | + | + | − | − | − | − | − | − |
| | β-Phenylethyl alcohol 0·6 | + | − | − | − | − | − | − | − |
| | Hydroxybenzoates 0·16 | + | + | + | + | + | + | + | + |
| | Hydroxybenzoates 0·106 | + | + | + | + | + | + | + | + |
| | Hydroxybenzoates 0·053 | + | + | + | + | + | + | + | + |
| | Hydroxybenzoates (Nipa 82121) 0·12 | + | + | + | + | + | + | + | + |
| | Hydrozybenzoates (Nipa 82121) 0·08 | + | + | + | + | + | + | + | + |
| | Hydroxybenzoates (Nipa 82121) 0·04 | + | + | + | + | + | + | + | + |
| | No preservative | + | + | + | + | + | + | + | + |
| Atropine Sulphate, 0·5 per cent. | Chlorocresol 0·1 | + | − | − | − | − | − | − | − |
| | Chlorocresol 0·03 | + | + | + | + | − | − | − | − |
| | Chlorbutol 0·5 (dissolved without heat) | + | + | − | − | − | − | − | − |
| | Chlorbutol 0·5 (dissolved with heat) | + | + | + | + | + | + | + | + |
| | Thiomersalate 0·005 | + | + | + | + | + | + | − | − |
| | β-Phenylethyl alcohol 0·5 | + | + | − | − | − | − | − | − |
| | β-Phenylethyl alcohol 0·6 | + | − | − | − | − | − | − | − |
| | Hydroxybenzoates 0·16 | + | − | − | − | − | − | − | − |
| | Hydroxybenzoates 0·106 | + | + | − | − | − | − | − | − |
| | Hydroxybenzoates 0·053 | + | + | + | + | + | + | + | + |
| | Hydroxybenzoates (Nipa 82121) 0·12 | − | − | − | − | − | − | − | − |
| | Hydroxybenzoates (Nipa 82121) 0·08 | + | − | − | − | − | − | − | − |
| | Hydroxybenzoates (Nipa 82121) 0·04 | + | + | + | − | − | − | − | − |
| | No preservative | + | + | + | + | + | + | + | + |
| Eserine Salicylate, 0·25 per cent. | Chlorocresol 0·1 | − | − | − | − | − | − | − | − |
| | Chlorocresol 0·03 | + | + | + | + | + | + | + | − |
| | Chlorbutol 0·5 (dissolved without heat) | + | + | − | − | − | − | − | − |
| | Chlorbutol 0·5 (dissolved with heat) | + | + | + | + | + | + | + | − |
| | Thiomersalate 0·005 | + | + | + | + | + | + | − | − |
| | βPhenylethyl alcohol 0·5 | + | + | + | + | − | − | − | − |
| | β-Phenylethyl alcohol 0·6 | + | − | − | − | − | − | − | − |
| | Hydroxybenzoates 0·16 | + | + | + | − | − | − | − | − |
| | Hydroxybenzoates 0·106 | + | + | + | + | + | − | − | − |
| | Hydroxybenzoates 0·053 | + | + | + | + | + | + | + | + |
| | Hydroxybenzoates (Nipa 82121) 0·12 | − | − | − | − | − | − | − | − |
| | Hydroxybenzoates (Nipa 82121) 0·08 | + | − | − | − | − | − | − | − |
| | Hydroxybenzoates (Nipa 82121) 0·04 | + | + | + | − | − | − | − | − |
| | No preservative | + | + | + | + | + | + | + | + |

infected 12 times. On one occasion the bottle containing atropine and thiomersalate kept at room temperature was found to be infected, but subsequent tests of the bottle showed it to be sterile again. The other solutions remained sterile throughout and when tried at the end of the experiment the atropine of all bottles gave good mydriasis in rabbits. Thus while maintaining sterility, the pharmacological effect of atropine was also retained in the presence of chlorocresol and thiomersalate.

## Chemical Compatibility

Benzalkonium chloride was found to be incompatible with argyrol, boric acid, silver nitrate, sodium fluorescein, and with the radicals nitrate and salicylate, e.g., pilocarpine nitrate and eserine salicylate (Macpherson and Wood[12]). McEwan and McMorran[17] tested the compatibility of chlorocresol, chlorbutol, and phenylmercuric nitrate with several substances: adrenaline, cocaine hydrochloride, ephedrine hydrochloride, homatropine hydrobromide, hyoscine hydrobromide, penicillin, pilocarpine and physostigmine. All these gave clear solutions with all the above preservatives, excepting homatropine and hyoscine, which were faintly opalescent, but which cleared on heating. These authors used very weak solutions of the drugs mentioned and it is possible that the precipitation with haloids did not become manifest. The B.P.C. and B.P. mention that these mercurial preparations cause precipitation with alkaloids. It seems therefore that the mercuric preparations are suitable mainly with eserine salicylate, pilocarpine nitrate and sodium fluorescein. Phenylethyl alcohol was tested by Brewer, Goldstein and McLaughlin[15] and was compatible with most of the ophthalmic solutions, and this is true of the *p*-hydroxybenzoates.

## Tolerance of the Eye

Chlorocresol in 0·1 per cent. solution and the *p*-hydroxybenzoates esters in 0·16 per cent. solution cause some burning sensation. Chlorbutol and phenylethyl alcohol have a slight anæsthetic effect which is of some advantage. The mercurial preparations are used in such dilution that no stinging or burning is caused by them. Sensitisation to mercury is extremely rare in that dilution.

## Conclusion and Summary

A number of substances were tested against *Ps. pyocyanea* (*Ps. æruginosa*) and their bactericidal concentration determined.

The quaternary ammonium compounds, although bactericidal, are not recommended because of their effect on the cornea.

The mercurial compounds:—thiomersalate 0·005 per cent. and phenylmercuric acetate and nitrate 0·005 per cent., are safe, and are recommended for eserine and sodium fluorescein, which are the most liable to pyocyanea contamination, also for methylcellulose eye-drops, which are liable to contamination by moulds. These compounds are not only powerful bactericides but are also fungicides. Chlorbutol in saturated solution (about 0·8 per cent.) is safe and recommended, but owing to the fact that the solution cannot be heated without detriment to the preservative its use needs great care. Chlorcresol 0·1 per cent. is safe, but in that concentration it causes smarting, and 0·03 per cent. kills pyocyanea within 24 hours. The *p*-hydroxybenzoates in 0·1 per cent. solution are reliable for most of the eye-drops used except fluorescein. Phenylethyl alcohol is safe in 0·5 per cent. concentration but much quicker in its action at 0·6 per cent., and further clinical trials are needed with this promising preservative.

For cortisone eye-drops 0·9 per cent. of benzyl alcohol, the one used in the original solution, is recommended.

Eye-drops used in hospital wards, outpatient departments and factory medical rooms, should be prepared with bactericidal preservatives, but for eye-drops used by individual patients, a bacteriostatic agent may be permissible. However, for reasons of safety and to make it as foolproof as possible a uniform procedure using a bactericidal preservative is recommended.

Up to now the National Formulary has recommended the use of bacteriostatic agents only. In view of the increasing incidence of eye infections reported by different authorities from many parts of the world, more stringent standards seem desirable.

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## References

1. Klein, *Brit. J. Ophth.*, 1931, **15**, 649.
2. McCullogh, *A.M.A. Arch. Ophth.*, 1943, **29**, 121.
3. Bignell, *Brit. J. Ophth.*, 1951, **35**, 419.
4. Rintelen, *Ophthalmologica*, 1951, **121**, 137.
5. Theodore, *Amer. J. Ophth.*, 1951, **34**, 1764.
6. King, Jnr., *ibid.*, 1953, **36**, 1388.
7. Theodore and Feinstein, *ibid.*, 1952, **35**, 656.
8. Morris and Truhlsen, *ibid.*, 1950, **33**, 357.
9. Haffley and Jensen, *Arch. Ophth. A.M.A.*, 1947, **37**, 649; Hogan, *Calif. Med.*, 1949, **71**, 414.
10. Hughson and Styron, *Amer. J. Ophth.*, 1949, **32**, 102.
11. Kedvessy, De Grosz and Szepes, *Brit. J. Ophth.*, 1950, **34**, 228.
12. McPherson, Jnr. and Wood, *Amer. J. Ophth.*, 1949, **32**, 675.
13. Klein and Millwood, *Brit. J. Ophth.*, 1953, **37**, 30.
14. Needham, *J. Hyg.*, 1947, **45**, 1.
15. Brewer, Goldstein and McLaughlin, *J. Amer. pharm. Ass., Sci. Ed.*, 1953, **42**, 584.
16. Ginsberg and Robson, *Brit. J. Ophth.*, 1949, **33**, 574.
17. McEwan and McMorran, *pharm. J.*, 1947, **104**, 260.

# THE ESTIMATION OF BARBITURIC ACID DERIVATIVES IN BIOLOGICAL MATERIAL FOR MEDICO-LEGAL PURPOSES

By Norman E. W. McCallum

*From the Department of Pathology, University of Melbourne*

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In medico-legal determinations of barbiturates in biological materials the usual method, the gravimetric Stas-Otto process, is time-consuming. A difficulty attending this type of examination is that the short-acting barbiturates may be almost completely metabolised (Brodie, Burns, Mark, Lief, Bernstein and Papper[1]). Hence, apart from those occasions when the examination may show the presence of such a small amount of barbiturate that it cannot account for the death, there are numerous occasions when this lengthy process gives negative results. Thus in its medico-legal application any method should possess the advantage of being rapidly conducted and, as its use may be extended to forensic cases admitted to hospital in a probable barbiturate coma, should be applicable to small volumes of body fluids.

Experience in this laboratory has confirmed the recognised disadvantages of some of the existing methods. Additional processes introduced in extraction techniques by some workers to eliminate the interfering factors have so lengthened the time of performance that, for medico-legal purposes, the methods have no advantages over large-scale procedures such as that of Valov[2].

## Nature of Interfering Factors

Gould, Mayo and Bowman[3] used an ether extraction process with a semi-micro continuous extractor and determined barbiturates spectrophotometrically by absorption in the ultra-violet region. They found that their method extracted other ultra-violet absorbing materials from serum, and for 100 samples of normal human serum (that is, serum supposedly free from drugs) the optical densities of the extracts varied from 0·040 to more than 0·200 at 240 m$\mu$. As the majority of sera gave these blank values in the range 0·100 to 0·150 with a mean at 0·130, these workers selected this last figure as the standard absorption value for serum. Walker, Fisher and McHugh[4] drew attention to the large amount of material, absorbing at 240 m$\mu$, which was extracted by the process of Jailer and Goldbaum[5] from 1 ml. of serum. This method involved a chloroform extraction of buffered plasma with subsequent transference of the thiopentone into sodium hydroxide solution. As these extracts from normal sera showed variation in absorption when they were in acid and alkaline medium, Walker *et al.*[4] discarded the method of direct extraction of the blood with chloroform in favour of a procedure giving a blank absorption which was independent of *p*H. In their method a tungstic acid filtrate of blood was extracted with chloroform and the latter was then extracted with 0·05 per cent. aqueous sodium hydroxide. The *p*H of the alkali was

adjusted to within a range of 9·0 to 10·5 and the absorption spectrum in the ultra-violet determined. The barbiturate was reported as the difference in absorption at 240 mμ between *p*H 10 and *p*H 2. The average recovery of pentobarbitone added to 2·5 ml. blood was 69 per cent. by this method, and determinations with other barbiturates returned a similar figure.

Tests were conducted in this laboratory with the various methods for the estimation of barbiturates in small samples of blood. Direct extraction using chloroform followed by sodium hydroxide was tried but was found highly unsatisfactory. On barbiturate-free material the absorption varied from sample to sample, the optical densities reaching values similar to those found by Walker *et al.*[4]. Their protein precipitation method was then tried both on normal sera and on sera to which phenobarbitone had been added. While the absorption in the case of normal sera was reduced so that maximum optical densities were in the vicinity of 0·10, the process was a long one and had the disadvantage that rather large losses of barbiturate were incurred, probably in the precipitation stage. It will be agreed by most of those engaged in the practice of forensic chemistry that calculations of concentrations which allow for losses incurred during analysis may not be well received in courts of law. In this country, at least, toxicological chemists prefer to report the actual amount estimated as the "minimum concentration" and sacrifice the amount lost in the extraction process rather than be placed in the position of explaining to a jury, uninformed in chemical procedure, why a large percentage has been added to the actual figure obtained in the estimation. In the method described by Walker *et al.*[4] the average recovery of barbiturate was 69 per cent., so that almost half this amount has to be added in each case. With phenobarbitone—probably the most commonly used barbiturate—a further correction of 13·6 per cent. is made for its own absorption in acid solution. For purposes other than forensic the method of Walker *et al.*[4] is reliable for the estimation of barbiturates but, to the forensic chemist, the above objection is real.

## Extraction of the Chloroform

In view of the foregoing it was decided to investigate whether the advantage of the Walker method—elimination of interfering substances—could be gained from a direct extraction process, thus reducing the loss of barbiturate. As indicated, the chloroform—sodium hydroxide extraction process is unsuitable for quantitative application. In addition, the *p*H of the extracting sodium hydroxide solution must be strictly controlled for reasons outlined by Walker *et al.*[4], and for further reasons described below.

In an effort to simplify the extraction procedure by elimination of the close control of the *p*H of the aqueous extracting phase, aqueous ammonia solution was tried as the transferring agent of the barbiturate from the chloroform. It was thought that the use of this weak base might also extract less of the interfering "chromogens". Tests showed that ammonia behaved in a manner similar to sodium hydroxide in the regions below *p*H 9 (Fig. 1). Above this value the behaviour of the two bases differed. In sodium hydroxide solution between *p*H 9 and 10·5 the extinction for a

given concentration of barbiturate is constant; above *p*H 11 the maximum at 240 mμ gradually falls and the peak moves in the direction of the visible spectrum. Walker *et al.*[4] demonstrate a curve at *p*H 12 showing a maximum at 245 mμ with a decreased optical density, and they regard this as the barbiturate molecule having undergone a further change, probably due to a second stage of ionisation. Figure 2, however, shows that this is only the beginning of the change as the alkali concentration increases. The maximum moves through 245 mμ and 250 mμ to become stationary at 255 mμ. This maximum at 255 mμ is reached when the sodium hydroxide concentration is between 0·05 and 0·075N. On further increasing the alkali concentration through 0·1 and 0·5N the optical density at 255 mμ increases until at concentrations of 0·5N and above, the optical density is constant. This fact does not appear to have been previously reported, and the results described are shown in Table I.

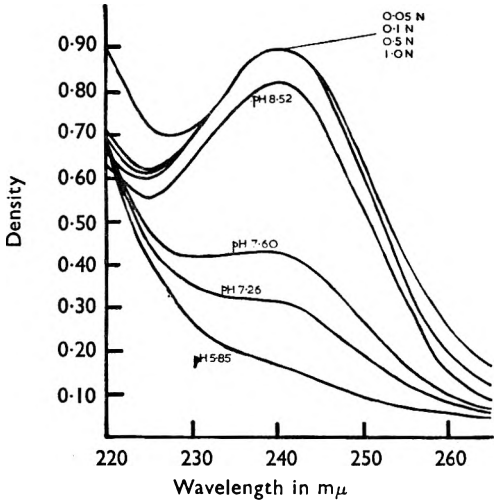


FIG. 1. Absorption curves for 20 μg./ml. of phenobarbitone. In all the curves above *p*H 8·52 the solutions are in ammonia at the concentration indicated.

Stuckey[6], in 1941, determined the absorption spectra of phenobarbitone and 1-methylphenobarbitone in 0·1N sodium hydroxide; he found the molecular extinctions for these compounds to be 8800 and 9000 respectively, but while in the case of phenobarbitone the maximum occurred at 256 mμ, that of 1-methylphenobarbitone occurred at 246 mμ. Methylation appears to have prevented the second ionisation stage and the consequent optical changes.

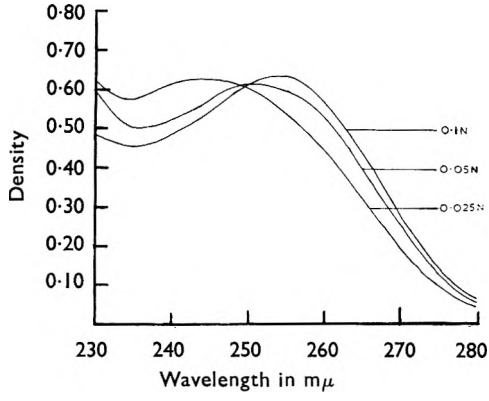


FIG. 2. Absorption curves for 20 μg./ml. of phenobarbitone in sodium hydroxide solution at the concentrations shown.

When aqueous ammonia solution was used as the extracting phase for chloroform it was found that for concentrations up to 15N the maximum

did not show any tendency to move from 240 mμ; the concentration of the extracting ammonia solution could thus safely vary between 0·1N (*p*H 11·1) and 1N (*p*H 11·4) without affecting either the magnitude of the optical density or the position of the maximum. In addition, these extracts could be left overnight without significant alteration.

TABLE I

| Concentration of sodium hydroxide solution | Wavelength of maximum (mμ) | Optical density |
|---|---|---|
| 0·025N | 245 | 0·654 |
| 0·05N | 250 | 0·638 |
| 0·1N | 255 | 0·662 |
| 0·25N | 255 | 0·680 |
| 0·5N | 255 | 0·694 |
| N | 255 | 0·694 |

## Quantitative Estimation

Since aqueous ammonia solution was a satisfactory extracting phase in the above respects, the relation between the maximum absorption values and the concentration was determined. Distilled water was put through the extraction process outlined below for blood, and the final 0·5N ammonia so obtained was used as the dissolving medium for phenobarbitone to produce curve 1 of Figure 3. This shows that the magnitude of the absorption at 240 mμ is proportional to the concentration of the dissolved barbiturate at least to a concentration of 25 μg./ml. With these solutions still in the silica cells of the spectrophotometer, 8N sulphuric acid was added to reduce the solutions to *p*H 2. After stirring, curve 2 of Figure 3 was obtained which, when corrected for dilution in the cells, showed that the acid absorption of phenobarbitone was regularly 15 per cent. of the alkaline value. It thus followed that aqueous ammonia solution was more suitable than sodium

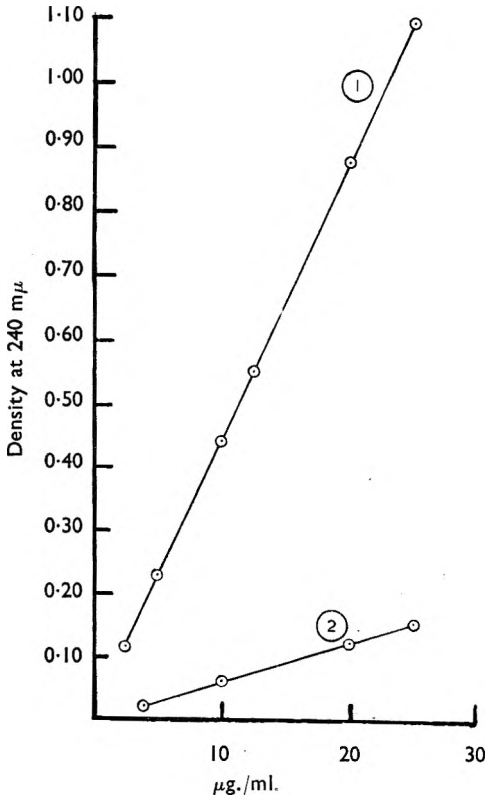


FIG. 3. Curve 1 shows the relationship between optical density and concentration of phenobarbitone in 0·5N ammonia. Curve 2 shows the same relationship when solutions are at *p*H 2.

hydroxide as the extracting phase for chloroform since the derivative is stable in the former solvent and the necessity to observe close control of *p*H is eliminated.

## Differential Extraction

The barbiturates are not extracted by the usual organic solvents from aqueous solutions of sodium hydroxide because they exist as salts. An attempt was therefore made, in order to avoid the precipitation loss found in the method of Walker *et al.*[4], to remove interfering substances by a process of differential extraction. Hence samples of barbiturate-free serum and urine were first made alkaline with sodium hydroxide and extracted with chloroform. This chloroform was discarded. The serum and urine samples were then acidified with hydrochloric acid and again extracted with chloroform.

The chloroform extract of the acidified material was then washed with a very dilute solution of sulphuric acid. Substances which partition fairly evenly between the organic and aqueous phases, and which would therefore be partially transferred to the chloroform during the acid extraction, were thus partially removed from the chloroform.

This chloroform from which nearly all interfering substances had, in the case of serum, been removed, was now extracted with aqueous ammonia solution and read on the spectrophotometer over the range 225 to 290 m$\mu$. In a series of 40 extractions of different barbiturate-free sera by this method, the optical densities varied from 0·012 to 0·078 at 240 m$\mu$. The majority were between 0·05 and 0·06 and only 2 were above 0·065. It was therefore evident that with such low and constant absorption in blank determinations there was no necessity with this method to read serum samples at *p*H 2 to obtain barbiturate absorption. Consequently in subsequent determinations the standard normal absorption of 0·050 was adopted for sera and was subtracted from the optical density of the barbiturate absorption at 240 m$\mu$.

TABLE II

| Phenobarbitone added to 2 ml. serum µg. | Recovery per cent. |
|---|---|
| 66·6 | 92, 90 |
| 133 | 88, 91 |
| 200 | 87, 88, 90, 92 |

The percentage recovery of barbiturate added to 2 ml. of serum was determined by simultaneously determining the absorption given by extracting 2 ml. of the same serum to which no barbiturate had been added. The recovery after subtraction of the blank absorption is given in Table II.

## Urine Extraction

The spectrophotometric determination of barbiturates in urine has received less attention in the literature than of that in blood. An objection to urine estimations may be that extractable metabolic products in which the barbiturate ring is still intact may contribute to the absorption. In the forensic field this may be of assistance in those instances where the blood barbiturate level is inadequate to account for the death, and may serve to support an opinion that a large amount was originally ingested.

Extraction of 1 ml. samples of normal urine by the chloroform-sodium hydroxide method gave absorptions in many cases which were beyond the capacity of the spectrophotometer to measure. When the volume was reduced to 0·2 ml., urine from patients known to have consumed large amounts of barbiturates gave absorptions in which the characteristic curve of the barbiturate was present but obscured. By applying the method described below, and adopting the Walker principle of measuring between *p*H 10 and 2 it was found that normal absorption could be reduced to measurable amounts (*ca.* 0·200–p·450), and subtraction of the *p*H 2 values restored the barbiturate curve.

## METHODS

### *Process for Blood*

*Procedure.* Three 50-ml. separating funnels are clamped on a burette stand one beneath the other. The reference blank is prepared by using 2 ml. of distilled water and following through the method.

1. 2 ml. of serum or plasma is placed in the top separating funnel and 1 ml. of 0·25N sodium hydroxide is added. This is shaken for 3 minutes with 15 ml. of chloroform A.R. When the phases have separated this chloroform is run off and discarded.

2. 1·5 ml. of 0·5N hydrochloric acid and 20 ml. of chloroform A.R. are then added to the aqueous phase in the funnel and the whole shaken for 3 minutes. After separation the chloroform is run through cotton wool held in a small filter funnel into the separating funnel below. The original aqueous phase is re-extracted with 10 ml. of chloroform A.R. and transferred in a similar manner to the second separating funnel.

3. This chloroform in the second funnel is shaken with 7 ml. of 0·1N sulphuric acid for 2 minutes, and the organic phase is run into the third funnel through cotton wool. 10 ml. of 0·5N ammonium hydroxide is added to this funnel and shaken for 3 minutes. The aqueous phase is transferred to a centrifuge tube in which it is centrifuged at 2000 r.p.m. for 2 minutes. The solution is then read in the spectrophotometer, against the standard blank, over the range 225 to 290 m$\mu$.

### *Process for Urine*

The volume of urine to be tested may vary from 0·2 ml. to 2 ml., depending on the concentration suggested by the blood level.

The procedure as for blood is used, except that 20 ml. of 0·5N ammonium hydroxide is used to extract the chloroform. Into the absorption cells is put 3·5 ml. of the ammonia extract and the absorption is determined. 7 ml. of the extract is titrated with 8N sulphuric acid to determine the volume necessary to reduce the *p*H of the solution to *p*H 2. The solutions are then treated with the appropriate volume of acid and stirred; the absorption at *p*H 2 is then determined. Correction must be made for the dilution in the cells and for the fact that, with phenobarbitone, there is a regular absorption by the acid form amounting to 15 per cent. of the alkaline form. The latter correction is unnecessary with the other common barbiturates.

## Summary

1. A method is described for the estimation of barbiturates in biological material.

2. The method avoids the extraction of substances giving undesirable blank values inherent in chloroform-sodium hydroxide extraction procedures.

3. The method simplifies the spectrophotometric determination of those procedures using sodium hydroxide as the vehicle for the barbiturate in the spectrophotometer.

4. The percentage recovery is about 90 per cent. and thus avoids, in forensic determinations, mathematical calculations for material which is not actually measured.


## References


1. Brodie, Burns, Mark Lief, Bernstein and Papper, *J. Pharmacol.*, 1953, **109**, 26.
2. Valov, *Industr. Engng Chem.*, 1946, **18**, 456.
3. Gould, Mayo and Bowman, *Fed. Proc.*, 1949, **8**, 295.
4. Walker, Fisher and McHugh, *Amer. J. clin. Path.*, 1948, **18**, 451.
5. Jailer and Goldbaum, *J. Lab. clin. Med.*, 1946, **31**, 1344.
6. Stuckey, *Quart. J. Pharmacol.*, 1941, **14**, 217.

# ANTICONVULSANT ACTIVITY

## Derivatives of Succinimide, Glutarimide, Thiazolidinedione and Methanol, and some Miscellaneous Compounds

By P. G. Marshall and D. K. Vallance*

*From British Schering Research Institute, British Schering Research Laboratories Ltd., Alderley Edge, Cheshire*

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Anticonvulsant activity has been associated with several types of compound, particularly the barbiturates, ureides, and hydantoins, and various members of these groups have proved of clinical value. The use of oxazolidinediones and in particular 3-allyl-5-methyl-oxazolidinedione, aloxidone[1], in the treatment of petit mal is also already well known, the latter being a particularly useful drug on account of the absence of the "glare phenomenon" usually associated with the clinical use of some other members of this group, e.g., troxidone. The anticonvulsant action of 5 : 5-diethyl- and 5 : 5-dimethyl-thiazolidinedione has also been reported[2]. More recently Chen, Portman, Ensor and Bratton[3] have described a number of α-phenylsuccinimides, several of which were found to be particularly effective anticonvulsants.

The purpose of the present paper is to describe screening tests for anticonvulsant activity which have been carried out on other derivatives of succinimide and some derivatives of the homologous glutarimide, one of which (Compound 415[4], α-methyl-α-phenylglutarimide) appears to be particularly active. In addition some derivatives of thiazolidinedione and of methanol, and some miscellaneous compounds have been investigated, and the results are reported here.

The method of test adopted was essentially the same as the maximal leptazol seizure pattern test used by Goodman, Swinyard, Brown, Schiffman, Grewal and Bliss[5]. Thus, an estimate of the relative activity of any given compound, compared with aloxidone, was obtained by determining the doses which would afford a certain degree of protection against leptazol-induced convulsions in mice. Groups of animals were premedicated with varying doses and, after a standard interval of two hours, which was chosen as a result of previous experience with troxidone and aloxidone, all the animals were injected intravenously with a "certainly convulsant dose" of leptazol. An estimate was thereby obtained of the dose required to abolish the hindleg extensor component of the maximal seizure in 50 per cent. of the animals (median protective dose, PD50).

### Experimental

Female albino mice of Schofield strain, weighing approximately 20 g. and starved from the previous day, were divided by random selection into groups of 6. The compounds for test were dissolved or suspended in a 5 per cent. solution of gum acacia, and serial dilutions were prepared.

*Present address, Biological Unit, The British Drug Houses, Godalming, Surrey.

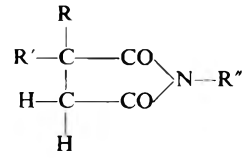
Volumes of 0·5 ml. per 20 g. of body weight were then administered orally, so that the animals received 506, 337, 225, and 150 mg. of the compound per kg. of body weight. Where the PD50 was found to be less than 150 mg./kg. the test was repeated with an appropriate reduction in the initial amount of the compound, the above geometrical progression of doses being continued.

After 2 hours, all the animals were given an intravenous injection of a previously determined "certainly convulsant dose" of leptazol (60 mg./kg.

TABLE I

SUCCINIMIDE DERIVATIVES

General formula:



| Compound | Substituent Groups | | PD50 mg./kg. | PD50 of aloxidone mg./kg. | Activity Ratio |
|---|---|---|---|---|---|
| | on C-atom | on N-atom | | | |
| Succinimide | — | — | >510 | 210 | <0·4 |
| 348 | — | allyl | 280 | 160 | 0·6 |
| 356 | phenyl | allyl | >510 | 220 | <0·4 |
| 379 | benzylidine | — | >510 | 130 | <0·3 |
| 384 | benzylidine | methyl | >510 | 170 | <0·3 |
| 386 | benzylidine | allyl | >510 | 170 | <0·3 |
| 385 | benzylidine | phenyl | >510 | 170 | <0·3 |
| 388 | benzylidine | benzyl | >510 | 140 | <0·3 |
| 387 | benzylidine | *cyclo*hexyl | >510 | 140 | <0·3 |
| 366 | *spiro-cyclo*hexyl | — | * | | |
| 362 | *spiro-cyclo*hexyl | methyl | * | | |
| 376 | *spiro-cyclo*hexyl | allyl | >510 | 150 | <0·3 |
| 369 | *spiro-cyclo*hexyl | hydroxyethyl | >510 | 150 | <0·3 |
| 363 | *spiro-cyclo*hexyl | phenyl | >510 | 150 | <0·3 |
| 365 | *spiro-cyclo*hexyl | benzyl | 340 | 150 | 0·4 |
| 364 | *spiro-cyclo*hexyl | *cyclo*hexyl | >510 | 150 | <0·3 |
| 370 | dimethyl | — | 280 | 150 | 0·5 |
| 374 | dimethyl | methyl | 130 | 150 | 1·1 |
| 375 | dimethyl | allyl | 220 | 150 | 0·7 |
| 367 | dimethyl | phenyl | 150 | 140 | 0·9 |
| 371 | dimethyl | benzyl | >510 | 150 | <0·3 |
| 368 | dimethyl | *cyclo*hexyl | >510 | 170 | <0·3 |
| 357 | methyl, phenyl | methyl | <150 | 220 | >1·5 |
| 408 | methyl, benzyl | — | 165 | 145 | 0·9 |
| 410 | methyl, benzyl | methyl | 220 | 145 | 0·7 |
| 409 | methyl, benzyl | allyl | >510 | 145 | <0·3 |
| 411 | methyl, benzyl | phenyl | >510 | 180 | <0·4 |

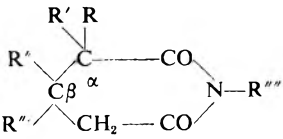
*Compound itself induced convulsions, even at dose level of 150 mg./kg.

of body weight). This dose had been found to induce clonic-tonic convulsions in all but very insensitive mice, and this was confirmed in every test by the responses observed in a control group of animals receiving no premedication. Only those mice showing the tonic extensor component of the leptazol-induced seizure were regarded as showing a positive response, and the percentage of animals protected in each group was calculated from the number failing to give this response.

In view of the small numbers of animals used, these values were then

## TABLE II

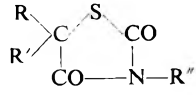
GLUTARIMIDE DERIVATIVES

General formula: 

| Compound | Substituent Groups | | | PD50 mg/kg. | PD50 of aloxidone mg/kg. | Activity Ratio |
|---|---|---|---|---|---|---|
| | on α-C-atom | on β-C-atom | on N-atom | | | |
| 415 | methyl, phenyl | — | — | > 70 | 130 | > 1·9 |
| 413 | methyl, phenyl | — | methyl | 110 | 130 | 1·2 |
| 394 | — | phenyl | — | 340 | 110 | 0·3 |
| 377 | — | phenyl | methyl | 380 | 150 | 0·4 |
| 383 | — | phenyl | benzyl | > 510 | 120 | < 0·2 |
| 381 | — | dimethyl | methyl | 510 | 130 | 0·3 |
| 391 | — | dimethyl | allyl | 240 | 130 | 0·5 |
| 395 | — | dimethyl | benzyl | > 510 | 150 | < 0·3 |

## TABLE III

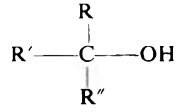
THIAZOLIDINE 2 : 4-DIONE DERIVATIVES

General formula: 

| Compound | Substituted Groups | | PD50 mg/kg. | PD50 of aloxidone mg/kg. | Activity Ratio |
|---|---|---|---|---|---|
| | on C-atom | on N-atom | | | |
| 354 | — | — | > 510 | 160 | < 0·3 |
| 353 | — | allyl | 290 | 150 | 0·5 |
| 351 | methyl | — | > 510 | 150 | < 0·3 |
| 350 | methyl | allyl | 300 | 210 | 0·7 |
| 349 | methyl | benzyl | > 510 | 210 | < 0·4 |
| 359 | dimethyl | — | 280 | 210 | 0·8 |
| 360 | dimethyl | methyl | 340 | 200 | 0·6 |
| 361 | dimethyl | allyl | > 510 | 200 | < 0·4 |

## TABLE IV

CARBINOLS

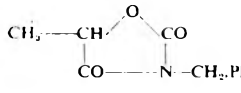
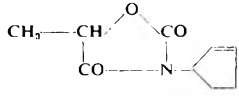
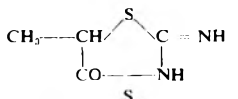
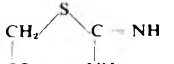
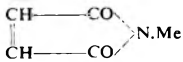
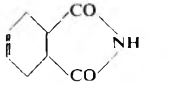
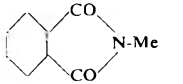
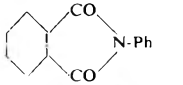
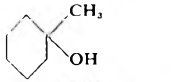
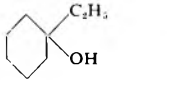
General formula: 

| Compound | Substituent Groups | | | PD50 mg/kg. | PD50 aloxidone mg/kg. | Activity Ratio |
|---|---|---|---|---|---|---|
| | R | R′ | R″ | | | |
| 396 | methyl | methyl | *n*-propyl | 150 | 180 | 1·2 |
| 404 | methyl | methyl | *iso*butyl | 150 | 280 | 1·9 |
| 389 | methyl | ethyl | ethyl | 150 | 140 | 0·9 |
| 397 | methyl | ethyl | *n*-propyl | 200 | 180 | 0·9 |
| 406 | methyl | *n*-propyl | *n*-propyl | 210 | 280 | 1·3 |
| 399 | ethyl | ethyl | ethyl | < 150 | 120 | > 0·8 |
| methylpentynol | methyl | ethyl | ethinyl | 60 | 140 | 2·3 |
| 333 | methyl | *n*-propyl | ethinyl | 55 and 67 | 120 and 110 | 2·2 and 1·6 |
| 339 | methyl | *iso*butyl | ethinyl | 190 | 160 | 0·8 |
| 343 | methyl | *n*-amyl | ethinyl | > 510 | 180 | < 0·4 |

treated where possible by the method of Thomson (described by Gaddum[6]) for obtaining estimates of median effective doses from the results of screening tests. The dose-response curve was smoothed by taking each

TABLE V

MISCELLANEOUS COMPOUNDS

| Compound | Formula | PD50 mg/kg. | PD50 of aloxidone mg/kg. | Activity Ratio |
|---|---|---|---|---|
| 378 | $Ph.CH_2.CO.NH_2$ | 340 | 130 | 0·4 |
| 382 | $Ph.CH_2.CO.NH.CH_3$ | 430 | 120 | 0·3 |
| 390 | $Ph.CH_2.CO.NH.CO.CH_3$ | >510 | 140 | <0·3 |
| 398 | $Ph.CH_2.CO.NMe.CO.CH_3$ | >510 | 150 | <0·3 |
| 347 |  | 210 | 160 | 0·8 |
| 403 |  | >510 | 150 | <0·3 |
| 352 |  | >510 | 160 | <0·3 |
| 355 |  | >510 | 210 | <0·4 |
| 393 |  | >510 | 150 | <0·3 |
| 400 |  | >510 | 120 | <0·2 |
| 402 |  | 340 | 150 | 0·4 |
| 401 |  | 510 | 150 | 0·3 |
| 407 |  | 190 | 160 | 0·8 |
| 392 |  | 170 | 130 | 0·8 |

set of 3 successive doses in turn, calculating the average effect in each set, and plotting these values against the middle doses of the sets. The median effective dose was then determined by inter-or extra-polation.

Where the screening tests indicated appreciable anticonvulsant activity, repeat tests were performed, using 2 to 4 dose levels and 10 to 30 animals

per dose. The values for the percentage protection were plotted against the respective doses of anticonvulsant on log-probability paper, and the PD50 and its confidence limits (P = 0·95) determined by the method of Litchfield and Wilcoxon[7].

In some instances preliminary toxicity tests were carried out in the usual manner, by oral administration of the compound as a solution or suspension in 5 per cent. acacia solution, and the number of deaths was recorded after 5 days.

## Results

In Tables I to V the results of the anticonvulsant screening tests are given, the values recorded being the estimated PD50 of the compound, the estimated PD50 of aloxidone in a parallel test, and the activity of the compound relative to that of aloxidone taken as unity.

### TABLE VI

DETAILED COMPARISON OF ANTICONVULSANT ACTIVITY OF SELECTED COMPOUNDS WITH THAT OF ALOXIDONE

| Compound | Median Protective Dose (confidence limits, P = 0·95, in brackets) | Activity Ratio |
|---|---|---|
| | mg/kg. | |
| aloxidone .. .. .. | 165 (135–201) | |
| 357 (α-methyl-α-phenyl *N*-methyl succinimide) .. | 69 (55-87) | 2·4 |
| aloxidone .. .. .. | 130 (100–170) | |
| 374 (αα′-dimethyl *N*-methyl succinimide) .. | 124 (92–167) | 1·0 |
| aloxidone .. .. .. | 162 (109–239) | |
| 333 (Methyl *n*-propyl ethinyl carbinol) .. .. | 89 (79–101) | 1·8 |
| aloxidone .. .. .. | 158 (133–188) | |
| 399 (Tri-ethyl carbinol) .. | 130 (97–174) | 1·2 |
| aloxidone | 149 (122–182) | |
| methylpentynol .. .. .. | 70 (57–86) | 2·1 |
| aloxidone .. .. .. | 168 (139–203) | |
| troxidone .. .. .. | 270 (206–354) | 0·6 |

### TABLE VII

COMPARISON OF ACUTE TOXICITY WITH THAT OF ALOXIDONE

| Compound | Number of deaths at | | | | | | LD50 g./kg. | Toxicity Ratio |
|---|---|---|---|---|---|---|---|---|
| | 0·7 g./kg. | 1·0 g./kg. | 1·5 g./kg. | 2·3 g./kg. | 3·4 g./kg. | 5·1 g./kg. | | |
| aloxidone .. | | 0/5 | 5/5 | 5/5 | 5/5 | 5/5 | 1·3 | |
| 348 .. | | 2/5 | 3/5 | 5/5 | 5/5 | 5/5 | 1·2 | 1·1 |
| aloxidone .. | | 0/5 | 2/5 | 5/5 | | | 1·5 | |
| 350 .. | | 2/5 | 4/5 | 5/5 | | | 1·0 | 1·5 |
| aloxidone .. | 1/10 | 3/10 | 6/10 | 9/10 | | | 1·4 | |
| 357 .. | 3/10 | 7/10 | 10/10 | 10/10 | | | 0·9 | 1·6 |
| aloxidone .. | | 1/5 | 3/5 | 5/5 | | | 1·5 | |
| 353 .. | | 3/5 | 5/5 | 5/5 | | | 1·0 | 1·5 |

## ANTICONVULSANT ACTIVITY

Table VI contains the results of the larger tests carried out on 5 selected compounds. Results obtained with troxidone have been included for comparison. The final column contains the Activity Ratio of the compound (aloxidone = 1·0), derived from the PD50 of the compound and the PD50 of aloxidone obtained in a parallel test.

## Discussion

Of the 66 compounds tested, only a few were found to be equal or superior to aloxidone in anticonvulsant potency, the majority being relatively inactive. Thus, none of the α-benzylidine derivatives of succinimide afforded any protection against leptazol at the dose levels usually employed. The same may be said, too, of all except one of the α-*spiro-cyclo*hexyl derivatives, and two of them were found actually to induce leptazol-like convulsions themselves. It might prove of value to investigate the possible use of these two (compounds 362 and 366) as analeptics. Varying degrees of activity were found among the αα-dimethyl succinimides, the most active being the *N*-methyl (confirmed by a large-scale test) and *N*-phenyl derivatives, which were similar in potency to aloxidone. Unsubstituted α-methyl- α-benzyl succinimide also appeared to be about as potent as aloxidone, but the introduction of *N*-substituents resulted in loss of activity. The most active succinimide tested was α-methyl-α-phenyl *N*-methyl succinimide which, in a large scale test, appeared to be 2·4 times as potent as aloxidone. It is also, however, 1·6 times as toxic. Chen *et al*[3] were the first to describe this compound as having a high degree of anticonvulsant activity.

In the glutarimide series, Compound 415 (α-methyl-α-phenylglutarimide) was found to be particularly active, and it has been the subject of considerable study, the results of which will be reported separately. Compound 413 (α-methyl-α-phenyl *N*-methylglutarimide) also appears to be more potent than aloxidone, but the β-phenyl-, and ββ-dimethyl-glutarimides were relatively inactive.

Thiazolidine 2:4-dione and its derivatives are all less active than aloxidone, the most potent (the 5:5-dimethyl derivative) having only about 80 per cent. of the activity of aloxidone (compare Hazard *et al.*[2]).

All the carbinols tested show a fairly high order of activity, methylpentynol (methyl-ethyl-ethinyl carbinol), in particular, being about twice as active as aloxidone. The anticonvulsant activity of methylpentynol has also been reported by P'an, Gardocki, Harfenist and Bauley[8] and P'an Markarian, McLamore and Bauley[9] and Reinhard, Kimura and Scudi[10]. Of the others, compound 333 (methyl *n*-propyl ethinyl carbinol) also appears to be about twice as active as aloxidone. It may also be noted here that other alcohols have been found to possess anticonvulsant properties e.g., *iso*propanol[11] and 2:2-diethyl-1:3-propanediol[12]. However, it must be borne in mind that many of the carbinols are potent narcotics, and, in addition, inspection of the results (unpublished) of toxicity studies with methylpentynol as reference compound indicates that many are more toxic than aloxidone. Compound 333, for example, appears to be at least twice as toxic.

None of the miscellaneous compounds listed in Table V was as active as aloxidone, and most showed little or no activity.

It is of some interest to endeavour to relate the chemical constitution of the various groups of compounds to the existence of anticonvulsant activity. The following compounds all exhibit considerable activity:—phenobarbitone, phenurone, diphenylhydantoin, aloxidone, a group of succinimide derivatives, and a glutarimide derivative (Compound 415 above) All of these contain the grouping

where R is alkyl or H and R′ and R″ are alkyl, phenyl, or hydrogen, and in the most active at least one substituent (R′ or R″) is phenyl. Phenobarbital is by far the most active, but it suffers from certain disadvantages that are well known, and further research in this field may yet bring to light a substance with more desirable overall therapeutic properties.

## Summary

1. The anticonvulsant activity of some derivatives of succinimide, glutarimide, thiazolidinedione, and methanol, and of some miscellaneous compounds has been investigated.

2. Earlier work on the activity of some derivatives of succinimide has been confirmed.

3. The most promising compounds appear to be methylpentynol (methyl-ethyl-ethinyl carbinol), methyl-*n*-propyl ethinyl carbinol, and α-methyl-α-phenyl-glutarimide. The last-named is to be the subject of more detailed investigation.

The authors acknowledge with pleasure the detailed chemical work involved in the preparation of the compounds, by Mr. P. A. McCrea, Dr. J. A. Baker, and Miss M. V. A. Chapman. They also thank Miss Needham for technical assistance.

## References

1. Brit. Pat. 626,971. Butter, *J. Neurol. Neurosurg. and Psychiat.*, 1952, **57**, 15.
2. Hazard, Cheymol, Chabrier and Smarzewska, *C. R. Acad. Sci., Paris*, 1948, **226**, 1850; 1950, **230**, 243.
3. Chen, Portman, Ensor and Bratton, *J. Pharmacol.*, 1951, **103**, 54.
4. Patent Pending.
5. Goodman, Swinyard, Brown, Schiffman, Grewal and Bliss, *J. Pharmacol.*, 1953, **108**, 428.
6. Gaddum, *Pharmacol. Rev.*, 1953, **5**, 87.
7. Litchfield and Wilcoxon, *J. Pharmacol.*, 1949, **96**, 99.
8. P'an, Gardocki, Harfenist and Bavley, *ibid.*, 1953, **107**, 459.
9. P'an, Markarian, McLamore and Bavley, *ibid.*, 1953, **109**, 268.
10. Reinhard, Kimura and Scudi, *ibid.*, 1952, **106**, 444.
11. Chu, Driver and Hanzlik, *ibid.*, 1948, **92**, 291.
12. Berger, *Proc. Soc. exp. Biol. N.Y.*, 1949, **71**, 270.

# ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

## CHEMISTRY

### ANALYTICAL

**Amino-acids, Paper Chromatographic Method for the Determination of.** A. L. Levy. (*Nature, Lond.*, 1954, **174**, 126.) The method is based upon the conversion of the amino-acids to their yellow 2:4-dinitrophenyl derivatives, followed by the separation of these compounds on a two-dimensional filter-paper chromatogram, and their subsequent elution from the paper and estimation at 360 m$\mu$. Dinitrophenylation is effected quantitatively by stirring an aqueous solution of the amino-acids (20 to 30 $\mu$M in 3 ml.) with a slight excess of 2:4-dinitroflurobenzene for 80 minutes at *p*H 9·0 and 40° C., the *p*H being maintained at this value throughout this period by intermittent additions of standard alkali. Excess reagent is then extracted with ether, the solution acidified, and the dinitrophenyl amino-acids extracted with ether. The aqueous solution, which contains dinitrophenyl arginine and $\alpha$-dinitro-phenyl histidine, is diluted to 10 ml. A 2 ml. aliquot of the ether solution and a 1 ml. aliquot of the water solution are applied to adjacent corners of an $18\frac{1}{4}$ by $22\frac{1}{2}$ inches sheet of Whatman No. 1 filter paper, which is then irrigated by the ascending procedure with a toluene-chloroethanol-pyridine-0·8N ammonia (5:3:1.5:3) mixture. The chromatogram is dried for 3 to 4 hours at 40° C. and the spots due to dinitrophenyl arginine and $\alpha$-dinitrophenyl histidine excised. The paper is then run on the second dimension by the descending procedure with 1·5M aqueous phosphate buffer. The spots are cut out, extracted with water and the optical density at 360 m$\mu$ measured. The dinitrophenol by-product does not interfere with the chromatography when the condensation is carried out as described above in a solution approximately 0·1M with respect to amino-acids. A. H. B.

**Codeine, Chromatographic Determination of.** G. C. McElheny, G. DeLamater and R. D. Rands. (*Analyt. Chem.*, 1954, **26**, 819). A chromatographic method of analysis is described which is applicable to opium, to intermediates, and to complex pharmaceutical preparations containing codeine. The procedure is based on the extraction of the opium with a saturated solution of sodium acetate, the nonphenolic alkaloids being isolated as a benzene solution and separated on a column of alumina with the aid of special developing solutions. By the method described thebaine, cryptopine, neopine, papaverine, and narcotine are eluted from the column before codeine and are separated completely from it, but not from one another. After deposition of the alkaloids on alumina from benzene solution a number of developing solutions containing *iso*propanol, chloroform, and benzene are used to elute the alkaloids; the effects of solvent changes on the elution are discussed. Tables are given showing the accuracy and precision as applied to the assay of opium. R. E. S.

**Cottonseed Oil, Detection of Trichloroethylene in.** G. A. Wiese and C. L. Jesina. (*Drug Standards*, 1954, **22**, 105.) In view of the increasing use of the toxic solvent trichloroethylene in the extraction of cotton-seed oil, the authors suggest that the U.S.P. XV should contain the following test for its presence. Add 2 ml. of A.R. pyridine to 2 ml. of 10 per cent, sodium hydroxide (U.S.P.)

solution in a test tube, which is then placed in a water bath at 90° C. After 5 minutes remove the tube from the bath and immediately add 1 ml. of the oil under test. A pink colour, developing in the pyridine layer within 20 minutes and varying from deep pink to faintly pink, indicates the presence of trichloroethylene. Comparison tubes containing: *a*, pressed cotton-seed oil, *b*, oil containing 1 in 100,000, *c*, 1 in 200,000 and *d*, 1 in 300,000 of trichloroethylene, treated as described above, are used to establish the concentration of the solvent.

J. R. F.

**Dihydrostreptomycin, Streptomycin and Framycetin and their Salts, Titration of, in Non-aqueous Media.** P. Pénau, E. Saïas and J. Ferdet. (*Ann. pharm. franç.*, 1954, **11**, 740.) The antibiotics were assayed by titration with perchloric acid in the presence of ethylene glycol and acetic acid. Large quantities of sulphate interfered with the colour changes of the indicators, unless previously removed with benzidine. The use of barium acetate for the removal of sulphate was not successful. The quantity of sulphate present in dihydrostreptomycin and streptomycin sulphates was first determined by titration with 0·1 N sodium hydroxide, using thymolphthalein as indicator (1/3 rd. of the sulphate was titrated). Sulphate in framycetin sulphate (an antibiotic from *Streptomyces decaris*) was determined by titration in the presence of pyridine. The antibiotic salts were dissolved in perchloric acid and ethylene glycol. So as to avoid an excess of the reagent, sufficient 0·05 M benzidine in glacial acetic acid was added to precipitate only 95 per cent. of the sulphate present. After allowing precipitation to occur, 2 drops of crystal violet indicator solution were added and the solution titrated with potassium phthalate. The quantity of dihydrostreptomycin, streptomycin or framycetin was calculated, an arbitrary figure being used for the molecular weight of framycetin. When titrating organic acid salts of the antibiotics, the benzidine solution was replaced by glacial acetic acid.

G. B.

**Fluoride, Colorimetric Determination of.** M. L. Nicholls and A. C. Condo. (*Analyt. Chem.*, 1954, **26**, 703.) Eighteen organic reagents, giving colour reactions with ferric iron in acid solution were studied and the wavelength of maximum absorption was determined; in addition a quantitative study was made of the effect of both *p*H and ferric ion concentration upon the ability of the fluoride ion to cause a fading effect. The fading effect was determined by measurement of the increase in transmittancy of the coloured complex at constant *p*H and constant ferric ion concentration as the fluoride ion concentration was increased. It was found that the ferric ion complexes of 3 reagents, resorcylaldoxime, 5-phenylsalicylic acid, and resacetophenone, gave approximately a 4 per cent. change in percentage transmittancy per 1 p.p.m. of fluoride at a *p*H of 2 to 3. The coloured complex of resorcylaldoxime was unstable on standing but the other two complexes were stable and gave reproducible and sensitive results for fluoride in concentrations from 0 to 6 p.p.m. Foreign ions which formed stable complexes with iron, such as citrate and tartrate, and also a high concentration of aluminium, interfered with the determination.

R. E. S.

**Glucose, Microdetermination of.** B. Mendel, A. Kemp and D. K. Myers. (*Biochem. J.*, 1954, **56**, 639.) A colorimetric micromethod for the determination of glucose is described, based on the formation of a bluish pink colour when one volume of a dilute solution of glucose is heated with 3 volumes of

96 per cent. w/w sulphuric acid; the intensity of the pink colour is proportional to the concentration of glucose. Details of the method are given together with light extinction curves for the solutions obtained; the final colour is measured at 520 mμ. The reaction is highly dependent on the concentration of sulphuric acid and appears to be specific for glucose, fructose and related carbohydrates. The concentration of glucose in the test solution is read from a standard curve; up to a concentration of 15 mg./100 ml., the relationship between the glucose concentration and colour intensity is linear, an extinction of about 0·160 at 520 mμ. being obtained with a aqueous solution containing 10 mg. glucose/100 ml The glucose content of blood can be determined after the blood has been deproteinised with a trichloroacetic acid solution containing silver sulphate. R. E. S.

***iso*Propanol in Dextran Solution, Determination of.** G. J. Frisone. (*Analyt. Chem.*, 1954, **26**, 924.) The method is a modification of the dichromate oxidation procedure of Stanley (*J. Assoc. off. agric. Chem., Wash.*, 1942, **25**, 693), the time per determination being shorter. By varying the concentration of sulphuric acid and of the potassium dichromate it was found possible to oxidise *iso*propanol to acetone within 5 minutes on a boiling water bath. Excess dichromate was then removed by the addition of sodium hydroxide, and the acetone was distilled into hypoiodite solution; excess iodine remaining was then titrated with standard thiosulphate. The accuracy of the method depended both on the primary distillation of the *iso*propanol from the dextran solution and on the secondary distillation of the acetone from the oxidation solution; the results obtained showed that the first distillation was not less than 98 per cent. complete. In the secondary distillation, analysis of two successive 60 ml. fractions of distillate showed that all the acetone was recovered in the first fraction. R. E. S.

**Sulphonamides, Paper Chromatography of.** P. Heinänen, L. Tuderman and L. Rämö. (*Farm. Notisblad.*, 1954, **63**, 66.) The method is a development of that previously described by the authors (*Farm. Notisblad.*, 1951, **60**, 84) using *n*-butanol saturated with 3 per cent. ammonia. Ehrlich's reagent is used for the detection of the spots. Sulphanilamide, sulphathiazole, sulphamethazine, sulphadiaizine and sulphacetamide may be separated and determined by extracting the single spots with ethanol and determining spectrometrically at 260 mμ. By this method it is possible to obtain a mean deviation of ± 1 to 3 per cent. G. M.

## GLYCOSIDES, FERMENTS AND CARBOHYDRATES

**Carbohydrates, Detection of.** R. U. Lemieux and H. F. Bauer. (*Analyt. Chem.*, 1954, **26**, 920.) A slightly alkaline (*p*H 7·2) spray reagent for chromatography is prepared by mixing 4 parts of 2 per cent. aqueous sodium metaperiodate and 1 part of 1 per cent. potassium permanganate in 2 per cent. aqueous sodium carbonate solution. The presence of a periodate- or permanganate-reducing substance on the paper results in the formation of a greenish-yellow spot, usually in a short period of time, the background retaining the permanganate colour for at least 1 hour at room temperature. The reagent can be used for the detection of 2 to 3 μg. of glucose, xylose, ascorbic acid, sorbose, maltose, cellobiose, and olefines; 5 to 8 μg. glucurono-lactone, 3-methylglucose, mannitol, erythritol, ethylene glycol, xylonolactone, and tartaric acid; 10 to 15 μg. of substances which reduce periodate slowly such as β-methyl glucopyranoside, sucrose, trehalose, pentaerythritol, 2:3:4:6-tetramethylglucose, 2:3-butanediol, and lactic acid. R. E. S.

**Erisimin, A Cardioactive Glycoside from *Erysimum canescens*, Roth.** V. V. Feofilaktov and P. M. Loshkarev. (*Doklad. Akad. Nauk*, SSSR, 1954, **94**, 709.) The properties of erisimin ($C_{29}H_{42}O_9 \cdot 2_2H_2O$), a cardiac glycoside separated from the herb *Erysimum canescens*, are described. It forms colourless needles, m.pt. 168 to 172° C. (decomp.), $[\alpha]_D^{20° C.} = +43{\cdot}48°$ (in ethanol); soluble in ethanol, in methanol and in acetone; insoluble in benzene, in light petroleum and in ether. It gave the characteristic colour reactions of the cardiac glycosides. No methoxy groups are present. The pharmacological action is similar to that of strophanthin; it contains 62,000 frog units per g., and in the cat assay the effective dose was 0·86 to 0·90 mg./kg. The triacetate ($C_{35}H_{48}O_{12}$) had m.pt. 230 to 232° C. The triacetate oxime ($C_{35}H_{49}O_{13}$) formed prismatic crystals with m.pt. 243 to 244° C. and $[\alpha]_D^{20° C} = 52{\cdot}19{\cdot}$ (ethanol). Hydrolysis of erisimin gave erisimidin ($C_{23}H_{32}O_6 \cdot 2C_2H_5OH$), m.pt. 161 to 164° C., soluble in ethanol and in methanol, less soluble in benzene and practically insoluble in water. By recrystallisation from hot chloroform-benzene, this was obtained free from ethanol with m.pt. 227 to 229° C. On heating with sodium hydroxide solution it formed the *iso*-aglycone, *iso*erisimidin, with m.pt. 210 to 213° C. The aglycone contains a $\beta$-oriented lactone ring at the C17 position, an angular CHO group at the C10, a secondary OH group at the C3, and a tertiary $\beta$-oriented OH group at the C14 position; the position of a third OH group was not established. The sugar component is of the digitoxose type. E. H.

## ORGANIC CHEMISTRY

**Analgesics, Stereochemistry of.** A. H. Beckett and A. F. Casy. (*Nature, Lond.*, 1054, **173**, 1231.) Certain isomers, (−)-methadone, (−)-ethyl-3-dimethylamino-1 : 1-diphenylbutyl sulphone, and (+)-3-dimethylamino-1 : 1-di (2′-thienyl)-but-l-ene which are the more analgesically active isomers of the respective enantiomorphic pairs, were shown to possess identical configurations related to that of D-(−)-alanine. Evidence is presented indicating that drugs exhibiting high analgesic activity have configurations complementary to that of specific receptor sites through which the pharmacological effect is mediated. The validity of the method by which Bick (*Nature, Lond.*, 1952, **169**, 755) assigned the absolute configuration of morphine as related to L-(+)-alanine is questioned. J. R. F.

**Azaserine, a New Tumor-inhibitory Substance, Isolation and Characterisation of.** S. A. Fusari, R. P. Frohardt, A. Ryder, T. H. Haskell, D. W. Johannessen, C. C. Elder and Q. R. Bartz. (*J. Amer. chem. Soc.*, 1954 **76**, 2878.) Detailed methods, involving chromatographic techniques used for the isolation of crystalline azaserine from culture broth filtrates of a *Streptomyces*, are described. Azaserine crystallises as light yellow-green needles from aqueous ethanol. It undergoes decomposition over a wide range (146 to 162° C.) upon melting. Dissolved in *p*H 7·0 phosphate buffer, it shows characteristic absorption in the ultra-violet region with one sharp, well-defined peak of $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 1140 at $\lambda$ 250·5 m$\mu$. The biological activity is destroyed and a hyperchromic shift to $\lambda$ 252 m$\mu$ ($E_{1\text{ cm.}}^{1\text{ per cent.}}$ 1230) occurs in 0·1N sodium hydroxide. There is a complete disappearance of ultra-violet absorption in 0·1N hydrochloric acid which is associated with a total loss of biological action and an evolution of nitrogen. The infra-red absorption curve is recorded. A sharp absorption band at 4·66 $\mu$ is present and, like the ultra-violet absorption peak at 250·5 m$\mu$, disappears completely upon acidification. The purity of azaserine was established by the Craig countercurrent technique. A. H. B.

## PLANT ANALYSIS

**Corchorgenin, A New Cardiac-active Aglycone from *Corchorus olitorius* L.** J. K. Chakrabarti and N. K. Sen. (*J. Amer. chem. Soc.*, 1954, **76**, 2390. A new cardiac-active aglycone, $C_{23}H_{32}O_6$, m.pt. 227° C., $[\alpha]_D^{23} + 90$ (ethanol) was isolated by chromatography of an ethanolic extract of the defatted seeds of *C. olitorius L.* grown in west Bengal. The aglycone is extremely bitter, soluble in ethanol, methanol and pyridine; sparingly soluble in water-chloroform and benzene, and insoluble in ether. It contains a hydroxyl group which can be acetylated to yield a monoacetate m.pt. 240 to 242° C. It exhibits a typical digitalis-like cardiac action which can be demonstrated with a concentration of $5 \times 10^{-6}$; this activity is practically identical with that of ouabain. In cats, its potency indicates that it is more active than either of the isomeric genins, corchortoxin or strophanthidin of *S. kombe.* It has been provisionally named "corchorgenin." A. H. B.

**β-Sitosterol and Corchorolic Acid, Isolation of.** G. Soliman and W. Saleh. (*J. chem. Soc.*, 1954, 1506.) The oil obtained by extraction of the seeds of *C. olitorius* with light petroleum was hydrolysed with ethanolic potassium hydroxide and β-sitosterol was obtained from the unsaponifiable matter. The latter was also isolated in a similar manner from the seeds of *C. capsularis.* The yellow substance obtained from the ethanolic extract of the seeds was a mixture of a phenol and an aliphatic hydroxy-acid, corchorolic acid, $C_{26}H_{52}O_3$, m.pt. 96° C. A. H. B.

## TOXICOLOGY

**Toxicology, Paper Chromatography in.** A. S. Curry and H. Powell. (*Nature, Lond.*, 1954, **173**, 1143.) A solvent system is reported for the chromatographic separation of basic organic compounds obtained in the toxicological analysis of viscera. The use of the modified Dragendorff reagent of Munier and Macheboeuf (*Bull. Soc. Chim. Biol.*, 1951, **33**, 846), provided an additional criterion to the $R_F$ value, giving a variety of shades of orange and red; 20 μg. of a substance could be easily detected. The solvent was a mixture of *n*-butanol (50 ml.): water (50 ml.): citric acid (1 g.), the upper layer being used. Before use the paper (Whatman No. 1) was dipped in a 5 per cent. sodium dihydrogen citrate solution, any excess liquid removed by blotting, and then dried at 60° C. for 25 minutes. The $R_F$ values are given for 28 basic substances. R. E. S.

# BIOCHEMISTRY

## BIOCHEMICAL ANALYSIS

**Calcium in Biological Materials, Estimation of.** J. G. Llaurado. (*J. clin. Path.*, 1954, **7**, 110.) A method is described in detail for the estimation of calcium, when mixed with other cations in biological materials, using a flame photometer burning coal-gas, an interference filter with a peak at 620 mμ, and an additional didymium glass to remove interference by sodium. The sample is treated with ammonium oxalate, the precipitate removed by centrifugation, dissolved by hydrochloric acid and submitted to flame photometry; a linear relationship between galvanometer deviations and calcium concentrations from 0 to 40 p.p.m. was shown. Tables of results are given and the sodium and potassium concentrations which can be present without affecting calcium

estimation are discussed. An interference effect of oxalic acid on calcium flame excitation is described for the first time, this being overcome by heating at 300° to 400° C., to destroy the oxalic anion. Results are given showing complete recovery of calcium in distilled water and in human serum. R. E. S.

**Isoniazid, Estimation of.** E. I. Short. (*Lancet*, 1954, **266**, 656.) A rapid and convenient method for the determination of isoniazid blood levels depends upon the reaction of the $-NH_2$ group of the hydrazide with 1 : 2-naphthoquinone-4-sulphonic acid and measurement of the colour (absorption maximum 455 m$\mu$) which develops. Since isoniazid does not penetrate the circulating red cells, samples of serum or plasma may be used instead of whole blood. A "protein-free" filtrate is prepared by adding to 4 ml. of plasma or serum, 5 ml. of distilled water, 4 ml. of a 9 per cent. solution of zinc sulphate and 5 ml. of 0·1 N sodium hydroxide solution, mixing after each addition, heating in a water bath for 3 minutes, cooling and filtering through Whatman no. 42 paper. To 9 ml. of the filtrate is added 1 ml. of a 0·1 per cent. w/v solution of 1 : 2-naphthoquinone-4-sulphonic acid The mixture is allowed to stand for about 1 hour before measuring the light absorption through an Ilford no. 601 or 621 filter. The measurement is made against a blank consisting of normal serum or plasma similarly treated by protein precipitation and reaction with 1 : 2-naphthoquinone-4-sulphonic acid. The result is read from a standard curve prepared with the aid of normal serum or plasma containing added isoniazid. Recoveries are satisfactory for clinical purposes, the average in these experiments being 100·5 per cent. The method is more accurate with the higher levels of isoniazid, the standard error of 6 estimates falling from ± 3·7 per cent. at 0·125 mg./100 ml. to ± 2·7 per cent. at 0·3 mg./100 ml. The method, with some modifications, may be applied to tissue, such as lung, or urine. G. B.

**Isoniazid, Estimation of, in Biological Fluids.** W. F. J. Cuthbertson, D. M. Ireland, W. Wolff and S. W. A. Kuper. (*Brit. med. J.*, 1954, **1**, 609.) The method depends on the reaction between isoniazid and picryl chloride (2-chloro-1 : 3-5-trinitrobenzene) to form a compound which may be measured absorptiometrically. For the determination in plasma (heparinised) the protein is first precipitated and the protein-free fluid employed. The extraction is carried out by a two-stage process using butanol-ether and 0·1 N hydrochloric acid. The picryl chloride is added to the acid extract, readings made against water in a Hilger absorptiometer, and the results calculated by comparison with a standard curve. This is prepared from readings obtained by applying the method to a series of known freshly prepared solutions of isoniazid in non-hæmolysed plasma. The method is also applicable to urine. The reproducibility of the method is satisfactory; the absorptiometer readings are linearly related to the amounts of isoniazid present within the range 0 to 20 $\mu$g. The standard deviation of a single estimation is about ± 10 per cent. *p*-Aminosalicylic acid was found to interfere with the assay, but not streptomycin. The method is not sensitive enough to detect any isoniazid in plasma 12 hours after the last dose of the drug. Isoniazid was estimated by this method in the plasma of 24 patients and in the urine of 9, and the results of the estimations are given. S. L. W.

**Methanol in Biological Fluids, Determination of.** M. Feldstein and N. C. Klendshoj. (*Analyt. Chem.*, 1954, **26**, 932.) In the method described, methanol is separated from biological material by diffusion in a standard Conway microdiffusion cell and is absorbed by a solution of sulphuric acid; it

is then determined quantitatively by oxidation to formaldehyde and subsequent reaction with chromotropic acid. Known amounts of methanol, added to blood and urine samples, showed recovery results ranging from 80 to 85 per cent. for less than 0·10 mg. of methanol. The incomplete recovery represents the diffusion equilibrium of methanol between the two solvents in the inner and outer compartments of the Conway cell; since, however, the equilibrium is constant under the conditions of the test, the methanol content can be calculated by applying a diffusion correction factor of 100/82·5 or 1·21. Using this factor, recoveries of methanol were shown to be 97 to 103 per cent. R. E. S.

**Salicylate in Biological Fluids, Determination of.** P. Trinder. (*Biochem. J.*, 1954, **57**, 301.) A rapid method for the determination of salicylate in biological fluids is presented, based on a reagent containing ferric nitrate, mercuric chloride and hydrochloric acid, which precipitates the proteins and simultaneously reacts with salicylic acid to give a purple colour. Details of the procedure, which can be completed in 5 minutes, are given. Recovery results on samples of serum, whole blood and urine, to which known amounts of sodium salicylate had been added ranged from 97 to 100·5 per cent.; the results were not affected by the presence of 100 mg. of phosphate ion, 20 mg. of bilirubin, 25 mg. of phenol, 10,000 I.U. of heparin, 1000 mg. of glucose or 1000 mg. of urea, per 100 ml. of serum. The blank values on normal serum and plasma samples are less than 1·1 mg. of salicylic acid/100 ml. R. E. S.

**Urinary 17-Ketosteroids, Determination of.** H. Werbin and S. Ong. (*Analyt. Chem.*, 1954, **26**, 762.) A modification of the Zimmermann reaction is described in which the absolute ethanol technique of Callow *et al.* (*Biochem. J.*, 1938, **32**, 1312) was used for the colour formation; the ethanol concentration of the pink solution was adjusted to 37 per cent. before extraction, and an improved apparatus is introduced for the extraction of the steroid chromogens. The modifications practically eliminated substances which interfered with absorption measurements at 400 mμ and gave an average recovery of 96 per cent. of the crystalline steroid when added to urine extracts. The absorption spectra of the chromogenic material from a number of urine extracts are given together with quantitative results for the concentration of 17-ketosteroids in neutral urine extracts. R. E. S.

## CHEMOTHERAPY

**8-Aralkyltheophyllines and Related Compounds.** G. P. Hager, J. C. Krantz Jr., J. B. Harmon and R. M. Burgison. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43** 152.) A number of 8-(substituted aralkyl) theophyllines were prepared in a search for compounds combining the hypotensive activity of 8-benzyltheophylline with greater solubility in water. They were obtained by fusion of 1:3-dimethyl-5:6-diaminouracil with the appropriate carboxylic acid and ring closure of the amide with sodium hydroxide solution or phosphorus oxychloride. The compounds generally showed a hypotensive effect when injected intravenously, but were inactive orally owing to lack of absorption from the gastrointestinal tract. They appeared to act directly on the arterial musculature. Polar solubilising groups of the electron releasing type did not decrease the activity of the compounds when substituted in the aralkyl group, but electron withdrawing groups decreased the activity slightly. The presence of a substituent at position 7 of the theophylline part of the molecule was found to be detrimental to hypotensive activity. G. B.

**8-(9-Fluorenyl)theophylline and Related Compounds.** G. P. Hager, C. T. Ichniowski and B. Misek. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 156.) 8-(2-Thienyl) theophylline was prepared by fusion of 2-thienylacetic acid with 1:3-dimethyl-5:6-diaminouracil and cyclisation of the amide with sodium hydroxide. 8-(3-Thienyl) theophylline was obtained similarly. 8-(9-Fluorenyl) theophylline was prepared by dehydrogenation of the Schiff base formed by the reaction of 9-formylfluorene with 1:3-dimethyl-5:6-diaminouracil. In experiments on anæsthetised dogs, 8-benzyl, 8-benzhydryl, 8-(2-thienyl), and 8-(3-thienyl) theophylline produced similar effects, the thienyl derivatives being slightly less active than the other compounds. Suitable doses produced a fall in blood pressure without an accompanying effect on the respiration. 8-(9-Fluorenyl) theophylline when similarly tested produced a pronounced transient fall in blood pressure and transient cessation of respiration followed by a longer compensatory rise in blood pressure, with respiratory recovery. 2-(9-Fluorenyl) 2-iminazoline and 2-(9-fluorenyl) 2-(9-fluorenylidenemethyl) 2-iminazolidine were also prepared but gave variable results during the preliminary pharmacological tests. G. B.

**Mercurial Diuretics.** L. H. Werner and C. R. Scholz. (*J. Amer chem. Soc.*, 1954, **76**, 2453.) Various mercaptans were combined with 3-hydroxy-mercuri-2-hydroxypropylcarbamylnicotinic acid sodium salt to produce compounds for investigation of the structural requirements of the thiol for maximal detoxification. Animal studies indicated that the polyhydroxyalkylthiols were highly effective in reducing the cardiac toxicity of mercurials; of these 1-thiosorbital appeared most promising. A number of mercurated compounds of different structures were prepared and combined with 1-thiosorbitol. A. H. B.

***iso*Nicotinyl Hydrazide, Mechanism of Action of.** D. S. Goldman. (*J. Amer. chem. Soc.*, 1954, **76**, 2841.) The *iso*nicotinyl hydrazide analogue of diphosphopyridine nucleotide (I) in which the nicotinamide moiety of the latter is replaced by *iso*nicotinyl hydrazide, was prepared by incubating diphosphopyridine nucleotide with beef spleen diphosphopyridine nucleotidase, and a large excess of *iso*nicotinyl hydrazide. Extinction coefficients for (I) at several *p*H's are given and the chemical and enzymatic activities are described. From the result obtained, it was considered that the antituberculous action of *iso*nicotinyl hydrazide might be due to the intracellular formation of an inactive pyridine nucleotide analogue with a concomitant reduction in cellular oxidative metabolism. A. H. B.

## PHARMACY

### NOTES AND FORMULÆ

**Antacid Capacity, A Method for Appraisal of.** A. M. Corrente. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 242.) The following method was used. Place 100 ml. of water in a beaker, maintain the temperature at 37° C. and stir continuously so that particles are only gently moved about. Add the test material (powder or tablets) and record the *p*H after 10 minutes. Add 10 ml. of 0·1 N hydrochloric acid, recording the *p*H after 45 seconds and 9 minutes, and continue the process, adding 10 ml. of acid every 10 minutes and recording the *p*H until the 45 second and 9 minute readings are the same, usually about *p*H 2. A variation involving the addition of pepsin (0·5 per cent. in 0·1 N hydrochloric acid) alters the pattern of response. The addition is made after the initial increments of the acid alone has reduced the *p*H to 6. Plot

the volume of acid against the two series of *p*H readings. Powders containing sodium bicarbonate with calcium carbonate or magnesium oxide showed a considerable capacity for neutralising acid, but within a range which is too alkaline, bearing in mind that the usual aim in antacid therapy is to maintain the *p*H within the range 3·0 to 3·5. Tablets of dried aluminium oxide showed an effect which varied with temperature and the presence of pepsin. At 37° C., in the presence of pepsin there was a large buffering capacity at low *p*H (2·0 to 3·0). Tablets of magnesium trisilicate with aluminium hydroxide gel were of limited buffering capacity. Some other commercial tablets were examined and the results tabulated. G. B.

**Dextran** (*New and Nonofficial Remedies*, *J. Amer. med. Ass.*, **154**, 154, 241.) Dextran is a water-soluble glucose polymer of high molecular weight obtained by the action of *Leuconostoc mesenteroides* on sucrose. The marketed product has an average molecular weight of about 75,000. It is administered intravenously as a 6 per cent. solution in isotonic sodium chloride to expand plasma volume and maintain blood pressure in the emergency treatment of hæmorrhagic and traumatic shock. The usual dose is 500 ml., given over a period of 15 to 30 minutes, and may be repeated when necessary if blood products are not available or indicated. For the treatment of hæmorrhage, the dose should be just sufficient to raise the systolic pressure to 80 to 85 mm. of Hg to avoid the production of further bleeding and dangerous dilution of the circulating blood. Dextran is excreted in the urine to the extent of 30 to 50 per cent., the remainder being metabolised. Almost no adverse reactions have been observed after repeated injections, although it appears to have a tendency to produce antigen-antibody type of reactions in some patients. These reactions are infrequent and mild if the dextran is refined and hydrolysed sufficiently to provide an average molecular size similar to that of serum albumin. Solutions of dextran do not require refrigeration. They should not be regarded as a substitute for whole blood or its preparations or for combating anæmia secondary to hæmorrhage, or severe wounds. G. R. K.

**Red Blood Cells, Preservation of.** P. L. Mollison. (*Brit. med. Bull.*, 1954, **10**, 27.) It is now generally assumed that the loss of viability of red cells stored at + 4° C. is due to the continuance of metabolic activity. This activity can be arrested by freezing, but the lowest practicable temperature at which blood can be stored without damage is − 2° C. and red cells stored at this temperature survive no better than those stored at + 4° C. Red cells mixed with glycerol can be frozen and thawed without lysis; there is a critical salt concentration which determines cell damage and in the presence of a glycerol concentration of about 23 per cent. w/v this salt concentration is not reached. It is already possible, using saline-glycerol as preservative, to maintain the viability of red cells for at least 6 months at − 79° C. but has not so far proved possible to preserve them for more than about 3 months at − 20° C. One way of improving preservation at − 20° C. is to leave part of the plasma with the red cells and to use a citrate-glycerol solution instead of saline-glycerol; this solution has a final sodium citrate concentration of about 1·5 per cent. w/v and a glycerol concentration of about 30 per cent. w/v. Red cells stored in this mixture for 3 months at − 20° C. undergo very little lysis and often have about a 90 per cent. survival after transfusion; they are agglutinated about as well as fresh red cells. Before the red cells can be transfused the glycerol must be removed by a continuous washing process such as described by Chaplin and Veall (*Lancet*, 1953, **264**, 218); a bottle of blood can be washed free of glycerol in about 2 hours. S. L. W.

## PHARMACOLOGY AND THERAPEUTICS

**Adrenaline, Noradrenaline and Dihydroergotamine, Action of, on the Human Uterus.** W. Garrett. (*Lancet*, 1954, **266**, 1060.) Adrenaline and noradrenaline stimulate strips of uterine myometrium taken from both pregnant and non-pregnant women; noradrenaline being about 3 times more potent than adrenaline. Noradrenaline also causes uterine contraction *in vivo* during late pregnancy and labour whereas adrenaline inhibits uterine rhythm. *In vitro* after dihydroergotamine adrenaline and noradrenaline no longer cause contraction. *In vivo*, during late pregnancy and labour the action of both amines is unaffected. M. M.

**Antiepileptics, Analgesic Effects of Clinically Useful.** E. A. Swinyard, D. L. Smith and L. S. Goodman. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 212.) Analgesic potencies were determined by measurement of the dose required to double the reaction time (withdrawal of the tail in response to a heat stimulus) in rats. Minimal neurological toxicity was assessed on the appearance of abnormalities in positioning sense, righting, gait and stance, muscle tone, equilibrium or other signs and was determined at the time of peak analgesic effect. The most powerful analgesic was phenobarbitone, followed by phethenylate, methylphenobarbitone, mephenytoin, phenytoin, paramethadione, phenacemide and trimethadione. All the antiepileptic drugs tested were more effective as analgesics than acetylsalicylic acid and less active than morphine. Neurotoxic action was observed to occur before analgesic activity, except for trimethadione and phenacemide. G. B.

**Blood Theophylline Concentration Following the Oral Administration of Theophylline Ethylenediamine and Theophylline *iso*Propanolamine.** A. E. Vivino. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 234.) Doses of theophylline with ethylenediamine and with *iso*propanolamine were administered to 25 normal subjects aged 20 to 28. A preliminary blood sample was taken for use in a blank determination. Samples were also drawn at intervals up to 10 hours after oral administration of 0·2 g. tablets of the drug, and assayed. Theophylline appeared in the blood within 15 minutes, and the concentration rose rapidly to a maximum (about 200 $\mu$g./100 ml. for theophylline with ethylenediamine and 350 $\mu$g./100 ml. for theophylline with *iso*propanolamine) at 1 hour. This high level was maintained for 4 hours, after which there was a gradual decrease in the blood theophylline level. Theophylline with *iso*propanolamine gave a more rapid rise in blood concentration and higher and more prolonged blood levels than theophylline with ethylenediamine. G. B.

**Polymyxin B Therapy of Meningitis.** J. P. Biehl and M. Hamburger. (*Arch. intern. Med.*, 1954, **93**, 367.) Details are given of 6 cases of meningitis following spinal anæsthesia or other procedures affecting the central nervous system. In 5 patients the infecting organism was identified as *Pseudomonas æruginosa*, while in the sixth it was *Proteus rettgeri*. The first two cases received penicillin, streptomycin and oxytetracycline, the second being given chloramphenicol and sulphadiazine in addition, but treatment was unsuccessful and both died. The third patient was given polymyxin B when his condition was critical but it did not prevent death. The fourth case was treated unsuccessfully with various antibiotics and sulphisoxazole but prompt recovery occurred when polymyxin B was given intrathecally. A prompt recovery also took place in the

fifth patient, who acquired a *Pseudomonas* infection while receiving intrathecal streptomycin for meningeal tuberculosis, when polymyxin B was added to the streptomycin injection. In the sixth patient the infecting organism, *Proteus rettgeri*, was sensitive to chloramphenicol as well as polymyxin B and the contribution of the latter to the patient's recovery may have been insignificant.

H. T. B.

**Polymyxin; Meningitis Treated with.** D. H. Trapnell. (*Lancet*, 1954, **266**, 759.) A case of *Pseudomonas pyocyanea* meningitis following a cerebrospinal fluid fistula in a boy aged 5½ is described. The causal organism was resistant to sulphonamides, penicillin, aureomycin, oxytetracycline and chloramphenicol, but was sensitive to polymyxin B. Treatment with polymyxin was commenced with a dose of 200,000 units intramuscularly 4-hourly and 40,000 units intrathecally 12-hourly for 3 doses and thereafter once daily. After a marked initial improvement, treatment was stopped on the 8th day, but was resumed 3 days later after a relapse. Polymyxin B was restarted in a dose of 200,000 units intramuscularly 4-hourly and 40,000 units intrathecally daily. Within 5 days temperature again became normal, and the systemic dose of polymyxin was decreased to 200,000 units 8-hourly, and four days later was further decreased to 200,000 units 12-hourly for the next 14 days. Apart from 4 days during which the daily intrathecal dose was halved, 40,000 units was given daily until the 37th day, 20,000 units being given for a further 2 days. Toxic effects included severe eosinophilia and xanthrochromia of the cerebrospinal fluid; other toxic effects observed were nausea and malaise, pain at the injection site, pain down the legs and in the back following intrathecal injections, and sacral œdema. These effects disappeared in spite of continued polymyxin therapy. In spite of its toxic effects, polymyxin is the drug of choice for pseudomonas meningitis. The case in question would probably have proved fatal but for this therapy.

S. L. W.

**Potassium Perchlorate, Treatment of Thyrotoxicosis with.** M. E. Morgans and W. R. Trotter. (*Lancet*, 1954, **265**, 749.) Potassium perchlorate inhibits thyroid function by preventing the thyroid from concentrating iodide. The object of this trial was to ascertain whether potassium perchlorate is effective, safe, and suitable for routine use in the medical treatment of thyrotoxicosis. 108 patients were treated; of these, 25 had had no previous treatment, 10 had previously completed a course of methylthiouracil but had subsequently relapsed, 64 were receiving methylthiouracil up to the time when perchlorate was started, and 9 were receiving methimazole. In the majority of the patients, potassium perchlorate in a dosage of 400 mg. daily was effective in controlling thyrotoxicosis. The rate of response appeared to be somewhat slower than with methylthiouracil, and 1 out of the 25 previously untreated cases was not completely controlled. When patients on maintenance doses of methylthiouracil were changed over to potassium perchlorate effective control of the thyrotoxicosis was maintained in all but 2 of 64 cases. The average dose necessary was from two to four times as great as that of methylthouracil. No toxic effects were seen in any of the patients, except for possible signs of gastric irritation in 2, both of whom had a previous history of dyspepsia. It is recommended as worthy of further trial, with the reservations that it is unsuitable for use in combination with iodides for pre-operative preparation, that in a few cases it has proved relatively ineffective, and that the possibility that it is a gastric irritant for some people has not yet been excluded.

S. L. W.

**Reserpine, Central Effects of, and their Antagonistic Reactions.** J. Tripod, H. J. Bein and R. Meier. (*Arch. int. Pharmacodyn.*, 1954, **96**, 406.) Reserpine, a recently isolated alkaloid from the root of *Rauwolfia serpentina* Benth shows a strong, long-lasting and characteristic depression of the central nervous system. This depression was compared with those of phenobarbital, sodium bromide and mephenesin and by the antagonisms which some drugs exert upon these effects. In its direct sedative action on mice, estimated as the shortening of the "fall-time" (the time the mouse can remain on a slowly rotating rod) reserpine equalled phenobarbital in its potency and was much more effective than sodium bromide and mephenesin; its duration of action was much longer than that of phenobarbital. Atropine prolonged sedation by the alkaloid and sodium bromide, but decreased that by phenobarbital. On the other hand, regitine decreased sedation by reserpine and phenobarbital, but prolonged that by sodium bromide. Reserpine antagonised the psychomotor stimulation induced in mice by pervitin (*d*-α-desoxy-ephedrine), caffeine, morphine, scopolamine and cocaine. Phenobarbital and sodium bromide showed synergism with all the stimulants save scopolamine, which was antagonised by both and cocaine, on which sodium bromide had no effect. Reserpine did not prevent convulsions in mice by strychnine, nicotine, picrotoxin and leptazol, but prevented audiogenic seizures. Phenobarbital, sodium bromide and mephenesin were effective anticonvulsants under each of these conditions. A miotic effect of long duration was observed with the alkaloid and morphine-induced miosis was increased, but reserpine also potentiated the mydriatic actions of atropine and scopolamine. Some antipyresis and a fall in body temperature were seen with the drug. Localisation of its site of action in the higher centres is discussed. G. P.

**Tetanus.** G. P. Wright. (*Brit. med. Bull.*, 1954, **10**, 59.) Experience with troops during the Second World War proved incontestably that prophylactic inoculation with tetanus toxoid can almost wholly eliminate tetanus under circumstances in which there is a serious risk of contracting the disease. The mechanism of active immunity (Boyd, *Lancet*, 1946, **250**, 113) may be regarded as functioning in two consecutive phases; firstly, neutralisation of toxin by pre-formed antitoxin (deriving from the last inoculation with tetanus toxoid) which is circulating in the blood stream; and secondly, neutralisation of toxin by antitoxin newly fabricated as the result of the toxin stimulus to the previously sensitised reticulo-endothelial system. Even if the antitoxin titre of the injured person at the time of infection were well below a protective level, if he possessed a previously experienced antitoxin-forming apparatus the stimulus of the toxin would suffice to evoke liberation of a large amount of neutralising antibody within a period much shorter than the usual incubation time of this disease. Although the disease is relatively uncommon in Great Britain, its distressing clinical course and high case-fatality rate encourage every effort for its prevention, especially when this can be achieved by so simple a prophylactic procedure. Since it is now well established that simultaneous prophylaxis with more than one antigen is not only feasible but possesses material advantages, there seems no valid reason why combined immunisation against tetanus and diphtheria should not be more widely employed as a public health measure. They need entail neither an additional nor a larger injection, for both the age at which it should be given and the time interval that separates the successive inoculations are the same as those at present current for diphtheria toxoid alone. Furthermore, such early immunisation would be effective at the time of life when the mortality from tetanus is at its peak. S. L. W.

# BACTERIOLOGY AND CLINICAL TESTS

***Cassia reticulata* Willd, Antibacterial Activity of.** H. W. Youngken and R. A. Walsh. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 139.) An extract was prepared by macerating the coarsely ground dried leaflets with a mixture of 1 volume of ethanol (95 per cent.) with 2 volumes of water, filtering and removing the ethanol by heating in a water bath. When tested by the F.D.A. agar plate method (modified for certain of the organisms), zones of inhibition were observed with *Escherichia coli*, *Alcaligenes fæcalis*, *Sarcina lutea*, *Proteus vulgaris*, *Salmonella typhosa*, *Bacillus megatherium*, *Pseudomonas æruginosa*, *Micrococcus pyogenes* and *Streptococcus pyogenes*. No inhibition of growth was observed for *Alcaligenes ærogenes*, *Serratia marcescens*, *Bacillus subtilis* and *Hæmophilus influenzæ*. Aqueous extracts of the leaves had a feebler antibacterial activity. Both aqueous and ethanolic extracts contained anthraquinones, emodin and chrysophanic acid. Some macroscopical characters of the crude drug are recorded. G. B.

***Mycobacterium johnei*, Cultivation of.** H. W. Smith. (*J. Path. Bact.*, 1953, **66**, 375.) This paper describes modifications of the liquid and solid media of Dubos (Dubos and Middlebrook, *Amer. Rev. Tuberc.*, 1947, **56**, 334) which have proved satisfactory for the cultivation of *Myco. johnei* and for its isolation from natural sources. Not only did bovine strains of *Myco. johnei* often grow more rapidly on these media than on Taylor's egg medium (Taylor, *J. Path. Bact.*, 1950, **62**, 647; 1951, **63**, 333), but the transparent nature of the solid medium was a great advantage, growth being much more easily detected. The fact that penicillin can be added to both the liquid and solid media to help control contaminants is another advantage not possessed by egg media.

S. L. W.

**Tetracycline, Bacteriological Properties of.** N. A. Diding. (*Svensk farm. Tidskr.*, 1954, **58**, 273.) The antibacterial effects of tetracycline, chlortetracycline and oxytetracycline were compared, by determining the minimal inhibitory concentrations in a serial dilution method. 2 ml. of the antibiotic solution (2400 μg./ml.) and 2 ml. of culture medium (Difco penassay broth) were mixed in the first tube, 2 ml. of the mixture transferred to a second tube containing 2 ml. of medium and so on. Each tube was inoculated with 1 drop of a 24-hour culture of the organism under test diluted 100-fold with sterile water. The tubes were incubated at 37° C. and observed after 24, 48 and 96 hours. The end-point was taken as the last tube showing no visible growth. The antibacterial effect of tetracycline was found to be of the same order as chlortetracycline and oxytetracycline when based on observations made after 24 hours' incubation. Thereafter the inhibitory concentration rose considerably on prolonged incubation, but the end-point was least affected for tetracycline, possibly because of the greater stability of this antibiotic. Tests were made against 9 organisms. Gram-positive organisms were inhibited by much lower concentrations than Gram-negative, except for a penicillin-resistant strain of *Staphylococcus albus*. Resistance was induced in 4 strains of bacteria by serial transfer in media containing increased amounts of the antibiotics. Induced resistance to one of the three substances was accompanied by a rise in resistance to either of the other two. G. B.

# BOOK REVIEW

*FRACTIONNEMENTS PAR SOLVENTS, by Maurice Vigneron 1st edition, pp.* 152 *(including* 61 *figures and* 6 *coloured plates). Vigot Freres, Paris*, 1954.

This book is of particular interest to those concerned with the large scale extraction and purification of pharmaceutical materials. In the words of Professor Charronat in the preface, it illustrates that " . . . le génie pharmaceutique, au sens récent du term, s'étend sur un vaste domaine scientifique." The work consists of three principal sections dealing with (i) industrial extraction of solids by solvents (ii) liquid-liquid extractions (iii) chromatographic fractionation. In each section an outline of theory is given but the book is primarily intended for the practical man. It is well illustrated with diagrams and photographs of industrial equipment in which the techniques described can be carried out. Most of the examples given are of a pharmaceutical nature and over 340 references to the literature are listed. In the first section, preliminary treatments of solid materials and choice of solvent to give optimum extraction, are discussed; there is an account of the elementary theory of liquid-solid extraction by simple and by multiple contacts; an outline is given of the counter-current method of extraction with numerous diagrams of industrial plant. The second section contains an elementary explanation of the theory of liquid-liquid extraction; counter-current, centrifugal and Craig methods of extraction are described, and the "theoretical plate" method of assessing extraction efficiency is explained. The third section deals with adsorption, partition, paper and ion exchange chromatography. The elementary theory of each method is presented and the relation between chromatographic bands and the maxima obtained in the Craig extractor, is stressed. This section is illustrated with coloured plates of chromatograms and with diagrams and flow sheets of industrial chromatographic plant. Some criticism can be made of the introduction to ion exchange. The treatment of ion exchangers in terms of their "solubilities" is not the most explicit theory for correlating their properties (p. 127); the general statement on p. 128 that the capacity of exchangers varies with *p*H. is not true for the strong anion and cation exchange resins; the author has not been selective in his lists of commercially available resins, they contain without distinction, modern, chemically simple exchangers and some of the older, more complex and generally less efficient, materials.

There are a few minor typographical errors in the text. For example, in the last equation on p. 58 a negative sign in the exponential has been omitted; two numbers have been misprinted in Table 13, p. 74; on p. 96 "l'absorbent" is twice used where "l'adsorbent" is intended; on p. 116 "cyanocobalamine"is an obvious misprint.

Considered as a whole, this is a valuable book, since it combines full descriptions of classical extraction methods with accounts of the newer chromatographic techniques. An English translation would be useful for students of Pharmacy.

L. SAUNDERS.

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