REVIEW ARTICLE

CHEMOTHERAPY: THE LABORATORY ASSESSMENT OF NEW DRUGS

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THE idea of the treatment of certain diseases by specific drugs is a very old one, although, since in most cases the treatment preceded the discovery of the causative organisms, the method was largely empirical. Thus, intestinal worms, according to Theophrastus,[1] were treated with male fern in the fourth century B.C. and the natives of Brazil used ipecacuanha for amœbic dysentery before the Spanish Conquest. Cinchona bark had been in use for the treatment of malaria for 200 years before Laveran[2] discovered the malaria parasite in 1880, and although *Spirochæte pallida* was not demonstrated as the causative organism of syphilis until 1905,[3] treatment of the disease with mercury is recorded as early as the sixteenth century.

The term chemotherapy for this type of treatment was introduced by Paul Ehrlich, who used it in a number of ways,[4,5] but always with the idea of specificity. He showed, for example, that organic compounds of arsenic were specifically active against certain spirochætal infections and certain organic dyes, such as trypan red, were active against trypanosomes.

Modern and systematic chemotherapy has developed from this conception of Ehrlich's of treatment by a drug, which, without unduly harming the host, attacks and causes the death of the parasitic cells.

Early chemotherapy was almost entirely confined to diseases caused by protozoa and spirochætes, and it was not until Domagk's discovery in 1935 of the chemotherapeutic activity of prontosil against experimental infections due to *Streptococcus pyogenes*[6] that this type of treatment was extended to bacterial infections. Since then, with the development of the sulphonamide drugs, penicillin, streptomycin and other antibiotics, bacterial chemotherapy has made phenomenal progress. In general, the mode of action differs in that the agent interferes with the basic metabolism of the bacteria, preventing normal growth. As a result, the concept of chemotherapy has to-day been modified to include suppression of the activity of the invading organism by the chemotherapeutic substance, thus enabling the normal defence mechanism of the body to deal with the infection.

In the search for new chemotherapeutic agents, 3 main lines of approach have been employed.

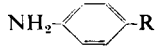
1. THE RATIONAL APPROACH

This resulted from the studies on bacterial metabolism by Fildes and his co-workers[7,8] and the interpretation by Woods of the mode of action of the sulphonamides.

Woods[9] drew attention to the structural similarity of sulphanilamide (II) and *p*-aminobenzoic acid (I), a substance essential for bacterial growth, and showed that the bacteriostatic action of sulphanilamide was antagonised by the essential metabolite. He concluded that the sulphonamides act by competing for an enzyme associated with the utilisation of *p*-aminobenzoic acid, thus blocking the use of the latter by the bacterial cell. Deprived of this essential metabolite, the bacteria become incapable of growth and reproduction and are readily destroyed by the natural defences of the body.

This concept of the substitution of a sulphonamide group for a carboxylic acid group in an essential metabolite producing a bacteriostatic effect, led to the preparation of a number of other metabolite analogues of similar structure. Some of these essential metabolites and their chemical analogues which have shown some inhibitory action *in vitro* are shown below (I to XI).

METABOLITE ANALOGUE



R = —COOH
p-Aminobenzoic Acid (I)

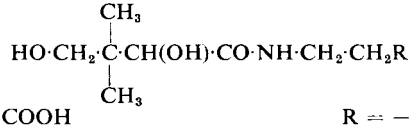
R = —SO_2NH_2
Sulphanilamide (II)

R = —COOH
Nicotinic Acid (III)

R = —SO_2OH
Pyridine-3-sulphonic Acid (IV)

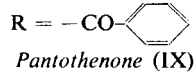
R = —$CONH_2$
Nicotinamide (V)

R = —SO_2NH_2
Pyridine-3-sulphonic Acid Amide (VI)

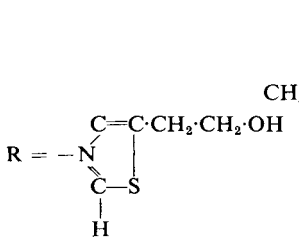


R = —COOH
Pantothenic Acid (VII)

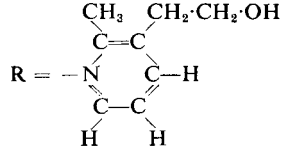
R = —SO_2OH
Pantoyl Taurine (VIII)



Pantothenone (IX)



Thiamine (*Aneurine*) (X)



Pyrithiamine (XI)

SOME ESSENTIAL METABOLITES AND THEIR SYNTHETIC ANALOGUES.

Although this approach would appear to be the most logical and has proved of value in contributing to the knowledge of bacterial metabolism and the mode of drug action, the results from the practical point of view have, on the whole, been disappointing.

This is probably due, firstly, to the extreme specificity of the substrate competition and the narrow line between growth inhibition and growth promotion. Thus nicotinic acid (III) or nicotinamide (V) is essential for the growth of various micro-organisms and pyridine-3-sulphonic acid (IV) acts as a growth inhibitor,[10] but some micro-organisms, such as *Proteus vulgaris*, which are inhibited by pyridine-sulphonic acid when nicotinic acid is the growth factor, are not inhibited when nicotinic acid is replaced by nicotinamide. Other organisms actually utilise pyridine-3-sulphonic acid as a growth factor.[11] Similar results are shown with pyridine-3-sulphonic acid amide (VI), which is bacteriostatic for organisms requiring nicotinamide as a growth factor but has little effect even at high concentrations on organisms capable of synthesising their own nicotinamide requirements.

Secondly, although the analogues were found to inhibit growth *in vitro*, it was found that they were not necessarily active *in vivo* because the metabolite itself was present in too high a concentration in the blood of the host. Pantoyl taurine (VIII), the sulphonic acid analogue of pantothenic acid (VII) was found to be bacteriostatic *in vitro* for those organisms which required pantothenic acid as a growth factor.[12,13,14] Chemotherapeutic action at non-toxic dose levels was demonstrated in hæmolytic streptococcal infections in rats but not in mice, and an explanation was afforded by the discovery of a high blood level of pantothenic acid in mice. In general, the rapid rate of excretion and the presence in the blood of the natural antagonist, pantothenic acid, render the analogue ineffective.[15] Variation in the substituent replacing the carboxylic acid group has produced other analogues, notably pantothenone (IX), with higher bacteriostatic activity.[16,17,18]

Pantothenic acid is also an essential metabolite for *Plasmodium*, the malarial parasite, and analogues have been found effective in experimental malarial infections.[19,20,21] The best is pantothenone, but it is not as active as the newer antimalarials, paludrine or chloroquine.

The third difficulty which has prevented these results proving of practical importance is that many essential metabolites are common to more than one cell, that is, they are essential to the host as well as to the micro-organism. An analogue which acts by substrate competition with a bacterial essential metabolite can be of value only when it does not appreciably affect the host. An illustration is afforded by thiamine (aneurine, vitamin B_1,X). The pyridine analogue, pyrithiamine (XI), inhibits the growth of organisms which are dependent on an external supply of thiamine, but it cannot be used chemotherapeutically, since, when administered in doses which would produce bacteriostatic blood levels, symptoms of vitamin B_1 deficiency appear in the host. Woolley and White[22] have shown that pyrithiamine produced, in rats and mice,

symptoms of thiamine deficiency which could be cured by giving a sufficient excess of the vitamin.

Similar results have been obtained with derivatives of folic acid. The analogue, 7-methyl-folic acid, inhibits the growth of *Streptococcus fæcalis* but when administered to rats a typical folic acid deficiency was produced which was reversed by the administration of the vitamin.[23]

A more promising application arises from the observation by Hertz[24] that the growth of the chick's oviduct in response to œstrogen administration requires an adequate intake of folic acid and in chicks given methyl-folic acid and treated with œstrogen the growth of the oviduct is markedly inhibited. This observation has been applied to the possibility of inhibiting the growth of malignant tissue. Folic acid has been shown to be essential for the growth of Rous sarcoma in chicks and growth can be inhibited by treatment with folic acid antagonists in doses which do not produce any symptoms of vitamin deficiency.[25] Similarly, the growth of transplantable mouse sarcoma has been inhibited by the folic acid antagonist, 4-amino-N^{10}-methyl-pteroylglutamic acid.[26] Work is continuing along these lines and it may well prove that while results from this line of approach have proved disappointing in bacterial chemotherapy, real progress may result in the chemotherapy of malignant disease.

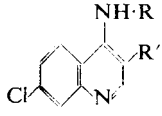
2. Preparation of Homologous Series of Compounds

The activity of synthetic chemical compounds is related to the molecular structure and, provided that the basic structure necessary for activity is maintained, it is possible to alter the specific properties by variation of other parts of the molecule. Examples of this type of development are numerous. Ehrlich himself, during his investigations on the organic arsenicals, prepared many series of compounds, the number 606 for Salvarsan giving some indication of the extent of his work. A second example is afforded by the development of the antimalarial drugs of the aminoquinoline series. Ehrlich[27] had shown, in 1890, that methylene blue had some curative effect on malaria, and the work of the Bayer research chemists in the preparation of methylene blue derivatives, in which the methyl groups were substituted by other alkyl groups, established that antimalarial activity was associated with a heterocyclic ring system. These results were applied to the quinoline ring system and two series of compounds with a substituted amino group in the -4 or -8 position have yielded a number of valuable antimalarial drugs. The relationship between the most important of these is shown below (XII to XVIII).

The 4-aminoquinoline derivatives originally prepared by German workers,[28] were re-assessed by the Americans during the war. They have activity approximately equal to that of mepacrine and possess the advantage of fewer toxic side-effects.[29,30,31,32] Sontoquin (XII) and chloroquin (XIII) act more rapidly in acute malaria, while camoquin (XIV) acts slightly less rapidly,[33] although there is some evidence that the latter may prove the best of the group.[34]

The 8-aminoquinolines have the distinctive property of killing the tissue stages of *Plasmodium vivax*. Pamaquin (plasmoquine) (XV), the

4-AMINOQUINOLINES



$R' = -CH_3$

$R = -CH(CH_3)(CH_2)_3N(C_2H_5)_2$

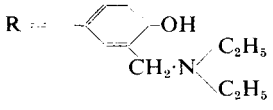
Sontoquin (XII)

$R' = -H$

$R = -CH(CH_3)(CH_2)_3N(C_2H_5)_2$

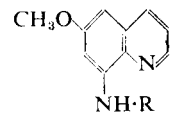
Chloroquin (XIII)

$R' = -H$



Camoquin (XIV)

8-AMINOQUINOLINES



$R = -CH(CH_3)(CH_2)_3N(C_2H_5)_2$

Plasmoquine (XV)

$R = -(CH_2)_5NH\,CH(CH_3)_2$

Pentaquine (XVI)

$R = -CH(CH_3)(CH_2)_3NH\,CH(CH_3)_2$

Isopentaquine (XVII)

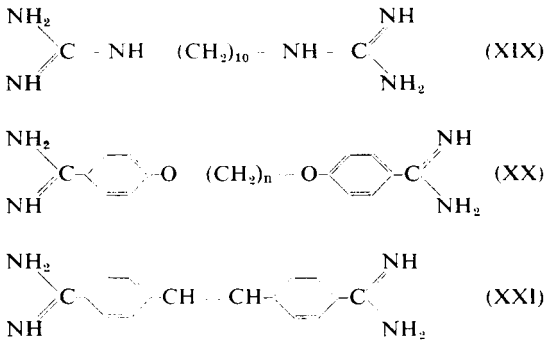
$R = -CH(CH_3)(CH_2)_3NH_2$

Primaquine (XVIII)

ANTIMALARIAL DRUGS OF THE 4- AND 8-AMINOQUINOLINE SERIES.

first of the aminoquinolines to be produced,[35] was found to be highly effective in combination with quinine in the treatment of malaria, but its toxicity precluded its general use.[36,37] Pentaquine (XVI) has similar properties and is somewhat less toxic,[38,39,40] while *iso*pentaquine (XVII) has equal activity and apparently even less toxicity.[41] The primary amine, primaquine (XVIII) is probably the most promising of the series, being the least toxic compound so far produced.[42]

Another valuable group of protozoal agents has resulted from the preparation of a series of amidine derivatives. The study of these compounds arose from the work of Lourie and Yorke on the mode of action of the guanidine derivative, synthalin (XIX). Synthalin was found to exert a therapeutic action on infections of *Trypanosoma brucei* in mice[43] and rats.[44] Guanidine derivatives lower blood sugar levels in animals[45] and Jancso suggested that the therapeutic effect of synthalin was an indirect one, due to the hypoglycæmia produced by the drug depriving the trypanosomes of the glucose necessary for their development.[43] To test this hypothesis, Lourie and Yorke[46] examined the *in vitro* effect

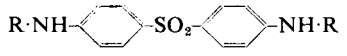


STRUCTURAL RELATIONSHIP BETWEEN DRUGS OF THE GUANIDINE AND AMIDINE SERIES.

of synthalin on trypanosomes and found it to have an extremely powerful trypanocidal action. Also it produced little hypoglycæmia in normal animals, except in doses large enough to cause liver damage. They concluded that the action of synthalin was a direct toxic effect on the trypanosomes and to confirm this they prepared a large number of guanidines, *iso*thioureas, amidines and amines with alkyl and alkylene chains.[47] Certain of the diamidines possessed powerful trypanocidal action both *in vitro* and *in vivo*. As a result of this work, a series of aromatic derivatives containing the amidine group were produced, some of which have proved of value chemotherapeutically.[48] The amidines are not specific in their action on protozoa, therapeutic effect having been demonstrated in trypanosomiasis, Leishmanial infections such as kala-azar and piroplasmosis such as *Babesia canis*. 4:4-Diamidinostilbene (XXI) was used effectively in kala-azar infections, but it was found to have a toxic action on nervous tissue,[49] due to the formation of a toxic dimer in solution. As a result, it has now been replaced by pentamidine (XX, n = 5) which has approximately the same activity, but is free from this toxic effect and is stable in solution.[50,51]

In the field of bacterial chemotherapy this method of development is well illustrated by the sulphonamide drugs. In 1935, Tréfoüel, Tréfoüel, Nitti and Bovet[52] demonstrated that the chemotherapeutic activity shown by certain dyestuffs containing the sulphonamide group[53] resided in the *p*-aminobenzenesulphonamide fragment. This substance, later known as sulphanilamide, was active against a number of Gram-positive organisms and by the introduction of substituents into the amide group it was possible considerably to extend its scope. Thus, sulphapyridine was found to be active against pneumococci[54] and sulphathiazole[55] and sulphadiazine[56] widened the range to include staphylococci. Sulphaguanidine, since it is only poorly absorbed, was found effective as an intestinal chemotherapeutic agent.[57] Later, sulphasuxidine and sulphaphthalidine, two sparingly soluble derivatives of sulphathiazole which are strongly hydrolysed to the active parent substance, were developed for the same purpose.

In addition to increasing the activity or specificity, modification of the molecule may be used to reduce toxicity. The activity of 4:4′-diaminodiphenyl sulphone (XXII) was reported by Buttle, Stephenson, Smith, Dewing and Foster in 1937,[58] but it was thought to be too toxic for therapeutic use.[59] Attempts to reduce the toxicity by the introduction of various substituents into the amino groups have been made in such



R = H	Diaminodiphenylsulphone (XXII)
R = $-CH_2SO_2Na$	Diasone (XXIII)
R = $-CH(SO_5ONa)\cdot(CHOH)_4\cdot CH_2OH$	Promin (XXIV)
R = $-CH(SO_2ONa)\cdot CH\cdot CH(SO_2ONa)C_6H_5$	Sulphetrone (XXV)

INTRODUCTION OF SUBSTITUENTS INTO THE DIAMINODIPHENYLSULPHONE MOLECULE. THE LD50'S OF THE RESULTING PRODUCTS ARE CONSIDERABLY GREATER THAN THOSE OF THE PARENT SUBSTANCE.

compounds as sulphetrone (XXV), promin (XXIV) and diasone (XXIII). These compounds are apparently less toxic than diaminodiphenyl sulphone, although there is some evidence that they may owe their activity to the liberation of the parent substance in the body.[60]

3. ANTIBIOTICS

Antibiotics are the products of growth of bacteria, yeasts, fungi and higher plants. Up to 1948, 133 substances had been described,[61] but, either because of their toxicity or because the antibacterial field which they cover was already adequately controlled by existing drugs, such as penicillin, few of them have been developed for medical use.

In addition to penicillin and streptomycin, 3 antibiotics which have proved of special value are aureomycin, chloramphenicol and terramycin. These 3 "wide-spectrum" antibiotics, all derived from moulds of the streptomyces group, are active against a number of Gram-negative organisms, rickettsias and viruses of the psittacosis and lymphogranuloma venereum groups, in addition to some Gram-positive organisms. All are well absorbed when given by mouth, but they are not entirely free from toxic side-effects, nausea, diarrhœa and stomatitis being the chief reactions. The sterilisation of the gut resulting from oral administration produces vitamin B deficiency, necessitating supplementary administration of the vitamin B complex.

Aureomycin is produced by *Streptomyces aurofaciens*, a new species of actinomyces isolated from the soil. Its preparation and properties are described in a series of papers in the Annals of the New York Academy of Sciences[62] and by Raistrick.[63] Satisfactory results have been reported in treatment with the drug against a variety of bacterial infections,[64,65,66] including gonococcal urethritis,[67] *Escherichia coli* vaginitis,[68] pneumococcal pneumonia and meningococcal septicæmia.[69] It is as effective as penicillin and the sulphonamides in pneumococcal and meningococcal infections,[70] but it is in the treatment of diseases due to Gram-negative organisms,

some viruses and rickettsias that the drug is proving most useful. Good responses have been obtained in brucellosis due to *Brucella melitensis*, *suis* and *abortus*,[71,72,73] and in the treatment of rickettsias of Q fever,[70] murine typhus[70] and rocky mountain spotted fever.[74] Wide use of aureomycin in urinary tract infections has been reported by Collins and Finland,[75] and it has proved of value particularly in infections due to Gram-negative organisms which were refractory to treatment with other antibiotics.[76] It is inferior to chloramphenicol in the treatment of typhoid and paratyphoid.

There is also some evidence that aureomycin may be of value in the treatment of amœbiasis.[77] It has the advantage of a dual effect, preventing secondary infection of the amœbic ulcers in addition to a direct amœbicidal action.[78]

Chloramphenicol was originally isolated from the culture fluid of a new strain of *Streptomyces* discovered in a sample of soil from Caracas, Venezuela in 1947[79] and given the name chloromycetin. It was prepared independently from a *Streptomyces* found in a compost heap at the Illinois Experimental Station.[80]

It is so far the only synthetically prepared antibiotic. The constitution and synthesis were described by Sweet and his co-workers in a paper to the American Chemical Society on March 29, 1949[81]; and Raistrick discussed its constitution at about the same time.[82] Chemically it is (1)-ψ-1-*p*-nitrophenyl-2-dichloroacetamidopropane-1:3-diol. The synthetic product has been found as effective as the natural one both experimentally and clinically.[83] The bacterial range is essentially similar to that of aureomycin and it appears to be equally effective in scrub typhus,[84] epidemic typhus,[85] Shigella enteritis,[86] brucellosis[87] and rocky mountain spotted fever,[88] and more effective and proving of particular value in the treatment of whooping cough[89] and typhoid fever.[90,91,92,93] One of the disadvantages in the latter case is the high relapse rate, but there is now some evidence that this can be reduced by treatment with an interrupted instead of a continuous course of the drug.[94]

Prolonged toxicity tests in dogs produced only a transient anæmia in animals given the drug for long periods by injection but not when given by mouth, and all the earlier clinical reports where oral administration was employed, emphasised the absence of toxic side-effects. More recently, however, reports have come from the United States that chloramphenicol has been found to cause aplastic anæmia which is often fatal.[95] These results serve to emphasise, first, the remarkable latent period which may elapse before the toxic effects of new drugs are recognised, and secondly the great care with which these new antibiotics should be used.

Terramycin, the newest of the 3 drugs, is derived from the soil organism, *Streptomyces rimosus*, and was introduced[96] in 1950. Its activity is similar to that of aureomycin and it is also readily absorbed from the gastro-intestinal tract, producing similar blood levels, although it differs in that increased dosage produces a marked increase in blood concentration.[97] Urinary excretion is also similar, although somewhat

higher urine concentrations at comparable dose levels are found with terramycin.[98]

Although it is too early for a final assessment of the value of the drug, it appears that it will be found as effective as aureomycin. Good results have been obtained with urinary tract infections, pertussis, pneumonia, bacillary dysentery, tonsilitis, erysipelas, typhoid fever[99] and rocky mountain spotted fever.[100,101] It is also reported to be efficacious in the treatment of amœbiasis.[102]

Two other groups of antibiotics which are of interest are bacterial in origin, being produced from various strains of *Bacillus subtilis* and *polymyxa*. Both groups are polypeptide in structure.[103,104,105]

Organisms of the *subtilis* group have produced several antibiotics, the most important of which is bacitracin.[106] It is effective chemotherapeutically in clostridial and streptococcal infections, and has been used successfully in the treatment of pneumococcal pneumonia.[107] It is, however, nephrotoxic, and its chief value therefore is as a local antibacterial agent.

Five closely related polymyxins have been isolated from different strains of *Bacillus polymyxa*[108,109,110] which are of interest because of their selective action against Gram-negative organisms. They are powerful chemotherapeutic agents against susceptible urinary tract infections, such as *E. coli* and *Ps. æruginosa* and, when given orally, are active intestinal antiseptics against Shigella and Pseudomonas infections.[111,112] Although they are not free from toxic effects, they have proved useful in the treatment of infections which have not responded to other less toxic drugs or antibiotics and also in the local treatment of wounds and burns.[113]

The Systematic Examination of Potential Chemotherapeutic Agents

The systematic examination of new compounds should include, in addition to *in vitro* tests for chemotherapeutic activity, determination of the acute toxicity, chronic toxic effects with continued dosage, *in vivo* activity where possible, and measurements of the rates of absorption and excretion after various routes of administration. Although the final assessment of any drug must be its therapeutic efficiency in man, such experiments with laboratory animals are of value in showing whether the activity is liable to be destroyed by plasma or other body fluids, indicating the dosage which may reasonably be employed and any potential danger of toxic side-effects.

The following account is illustrated by examples of antibacterial substances, but the methods are essentially similar whatever type of infection it is hoped to control.

In Vitro Activity

A rough qualitative idea of the antibacterial activity may be conveniently obtained on a nutrient agar plate. The plate is divided into a number of sections, a cup cut centrally and the substance to be tested

placed in the cup. The sections are streaked with cultures of different organisms and the plate incubated at 37° C. for 24 hours. The amount of inhibition gives a rough estimate of the relative activity of the substance against the different organisms.

A more exact quantitative estimate of *in vitro* activity may be determined by a serial dilution method using a series of tubes containing a culture of the organism diluted with nutrient broth to give the same definite concentration in each tube. Successive dilutions of the test

TABLE I

POLYMYXIN AND *Salmonella typhi*

Activity with varying inocula

Polymyxin	Organisms per ml.	Concentration of polymyxin—"A" units per ml.							
		20·0	10·0	5·0	2·5	1·25	0·62	0·31	0·16
A	10^7	−	−	−	+	+	+	+	+
	10^5	−	−	−	+	+	+	+	+
	10^3	−	−	−	−	+	+	+	+
	10^1	−	−	−	−	−	−	+	+
B	10^7	−	−	−	−	+	+	+	+
	10^5	−	−	−	+	+	+	+	+
	10^3	−	−	−	−	−	−	+	+
	10^1	−	−	−	−	−	+	+	+
C	10^7	−	−	+	+	+	+	+	+
	10^5	−	−	+	+	+	+	+	+
	10^3	−	−	−	+	+	+	+	+
	10^1	−	−	−	−	−	+	+	+
D	10^7	−	−	−	+	+	+	+	+
	10^5	−	−	−	−	+	+	+	+
	10^3	−	−	−	−	+	+	+	+
	10^1	−	−	−	−	−	+	+	+
E	10^7	−	−	+	+	+	+	+	+
	10^5	−	−	−	+	+	+	+	+
	10^3	−	−	−	−	+	+	+	+
	10^1	−	−	−	−	−	+	+	+

substances are added and the tubes incubated. The minimum concentration required to inhibit growth is thus determined and the antibacterial action of the substance against a wide range of pathogenic organisms may be obtained in this way. Interpretation of these results in conjunction with toxicity tests and absorption studies shows whether the required concentration for inhibition at the site of infection is likely to be reached.

The *in vitro* activity is controlled to a greater or lesser extent by the size of the inoculum, so in the determination of antibacterial activity by the serial dilution method, relatively small inocula are used. Some measure of the efficiency of the agent may be obtained by determining the relationship between the inhibitory concentration and the size of the inoculum. Table I gives the results of such a test with the polymyxins, 5 antibiotics obtained with various strains of *Bacillus polymyxa* and varying inocula of *Salmonella typhi*. In general, the heavier the infection, the greater is the concentration of drug necessary to inhibit growth and the steeper the slope of the curve relating inhibitory concentration and inoculum concentration, the more efficient is the antibacterial substance.

In the case of penicillin, it is almost perpendicular, relatively low concentrations of the drug being effective over a wide range of inoculum concentration.

In Vivo Tests

It does not necessarily follow that because a substance is active in inhibiting bacterial growth in the test tube, it will be similarly effective in the active disease. The general *in vitro* antibacterial tests are followed by more specific *in vivo* tests using the organisms against which the substance has been shown to be most effective. Animals are infected with several times the lethal dose of the pathogenic organism and are then given a course of treatment with the drug at varying dose levels. A control group of animals are infected but not treated. The number of animals surviving each day is recorded and the results assessed by calculating the average day survival rate, obtained by adding together the number of animals surviving on each day and dividing by the number of days. If possible the cause of death is confirmed by recovery of the infecting organism. Mice are generally the most convenient animals for these tests.

Table II compares the results of an *in vivo* test with the polymyxins and two types of organism. The *in vitro* and *in vivo* activity of these antibiotics has been described fully by Brownlee, Bushby and Short.[114,115] The infection obtained in mice with *Hæmophilus pertussis* is chronic, and observations must be continued for a longer period than in the test with *Salmonella typhi*, which results in an acute infection, the control animals showing ill effects and some even dying within 6 hours of infection.

The disease produced in animals is not necessarily comparable to that produced in man by the same organism, but the results may be of value in giving some indication of whether the drug is inactivated by plasma or other body fluids or whether it is destroyed so rapidly in the body that it will not reach a sufficiently high level in the blood to be therapeutically active.

Acute Toxicity

The second essential requirement of a good chemotherapeutic drug is that it should not be unduly toxic to the host. Some measure of the relative acute toxicity and the therapeutic efficiency may be obtained by calculation of the therapeutic index from the results of animal experiments.[116] The therapeutic index is numerically equal to the ratio of the median toxic lethal dose to the therapeutic dose, and the higher the therapeutic index, the greater is the margin of safety in the use of the drug. Penicillin has a very high therapeutic index, it is in fact the highest of any known drug and is many times the value for most of the sulphonamides.

The acute toxicity is measured in animals as the median lethal dose or LD50, the dose necessary to kill half the animals used in the test. Mice are the most convenient animals for the preliminary determination of toxicity. Groups of mice are given increasing doses of the compound and the number of deaths at each dose level is recorded. The minimum

TABLE II

In vivo TEST WITH (A) A CHRONIC AND (B) AN ACUTE INFECTION

A. Mice infected with *Hæmophilus pertussis*—intracerebrally 10,000 lethal doses

Drug: twice daily for 3 days

Dose units	Number of mice	Number of mice surviving on day							Average day survival
		2	4	6	8	10	12	14	
A 500	10	10	10	10	10	10	10	10	14·0
250	10	10	10	10	9	8	7	7	12·2
B 500	8	8	8	8	8	8	8	8	14·0
250	9	9	9	9	9	9	8	8	13·6
C 500	10	10	10	10	10	10	10	10	14·0
250	9	9	9	9	9	8	8	8	13·3
D 1000	10	10	10	10	10	10	10	10	14·0
500	8	8	8	8	5	4	3	3	9·8
E 500	9	9	9	9	9	9	7	7	13·1
250	10	10	10	10	7	6	6	6	12·2
Controls ..	9	9	9	8	4	—	—	—	6·6

B. Mice infected with *Salmonella typhi*– intraperitoneally 10,000 lethal doses in 5 per cent. mucin

Drug: twice daily for 4 days

Dose units	Number of mice	Number of mice surviving on day							Average day survival
		1	2	3	4	5	6	7	
1000	10	10	10	10	10	10	10	10	7·0
A 500	10	10	9	9	9	9	8	8	6·2
250	10	8	8	8	8	8	8	8	5·8
1000	10	10	10	10	10	10	9	9	6·8
B 500	10	9	9	9	9	9	9	9	6·5
250	10	6	4	4	4	4	4	4	3·0
1000	10	10	10	10	10	10	10	10	7·0
C 500	10	10	10	10	10	10	10	10	7·0
250	10	9	9	9	9	9	9	9	6·3
1000	10	10	10	10	10	10	10	10	7·0
D 500	10	10	10	10	10	10	10	10	7·0
250	10	9	8	8	8	8	8	8	5·7
1000	10	10	10	10	10	10	10	10	7·0
E 500	10	10	10	10	10	10	10	10	7·0
250	10	6	3	3	3	3	3	3	2·3
Controls ..	10	—	—	—	—	—	—	—	0·0

dose required to kill half the animals is then calculated graphically from the results.[117,118] The acute toxicity varies with the route of administration, generally the oral toxicity is about one-tenth the intravenous or intraperitoneal, and the toxicity following intramuscular or subcutaneous injection is usually somewhat less than by the intravenous or intraperitoneal route. Again, only indications of the probable toxicity of the compound in man can be obtained from animal tests.

It is not infrequently found that certain species are particularly susceptible to a certain type of compound and although it is possible to eliminate errors due to species variation to a certain extent by making determinations on a number of different species, the translation of results

from animals to man is difficult, because of such factors as differences in metabolic rate and surface area.

Absorption and Excretion Studies

The drug, to be effective chemotherapeutically, must be sufficiently absorbed to give a blood level which will ensure that a bacteriostatic concentration is attained at the site of infection. The speed with which this blood concentration is reached and the length of time for which it is maintained, determines the size and frequency of dose. Measurements of absorption and excretion rates help to supply this information.

Estimation. Since only minute concentrations are normally necessary to inhibit the growth of micro-organisms, the methods of estimation of the drugs must be extremely sensitive. In the case of antibacterial substances, a microbiological method can usually be employed, the degree of inhibition of a sensitive organism being compared with that produced by a standard concentration. Comparison may be made either by the plate and cup method or by the serial dilution method.

Although microbiological methods are extremely sensitive, they have the disadvantage that they are rather lengthy and their reproducibility is somewhat variable, so that where possible microchemical or physical methods have been developed.

Both aureomycin and terramycin can be estimated by physical methods depending on their ultra-violet absorption[119] or their fluorescence in solution.[120,121]

Microchemical methods are generally either colorimetric or dependent on a microtitration. An aromatic amino group is of frequent occurrence in synthetic drugs and several methods depending on the production of a dye after diazotisation of this group have been evolved. Bratton and Marshall,[122] for example, applied this reaction to the estimation of the sulphonamides. After treatment with sodium nitrite, the diazonium compound is coupled with *N*-(1-naphthyl)ethylenediamine, producing a pink dye, the colour intensity being dependent on the concentration of drug present. This method has been applied to a number of drugs, including the amino sulphones and some of their derivatives.[123]

A somewhat more specific reaction for certain aromatic amines described by Ehrlich, depending on the formation of a yellow compound with *p*-dimethylaminobenzylaldehyde, can also be applied to a number of drugs. It gives, for example, a sensitive and accurate method for the estimation of *p*-aminosalicylic acid.[124] It is also possible by using two or more of these reactions to evolve differential methods for the estimation of mixtures of similar drugs.[124]

By making use of various parts of the molecule, chemical methods of estimation have even been developed for a number of the antibiotics, which have, in general, a more complicated structure than synthetic drugs. Several such methods are now used for the estimation of penicillin and streptomycin.[125,126,127,128,129,130] Reduction of the nitro group followed by the Bratton and Marshall's diazo procedure has been applied to the estimation of chloramphenicol and its breakdown products.[131] Similarly,

estimation of the constituent amino-acids affords a chemical method for the evaluation of polypeptide antibiotics. This method has been applied to the polymyxins by estimation of their threonine content.[132] After acid hydrolysis, the threonine is oxidised by periodic acid to acetaldehyde in a micro-diffusion cell, the acetaldehyde is trapped as the bisulphite compound, which is estimated by a microtitration with iodine. Polymyxin B and C can be assayed colorimetrically by the estimation of their phenylalanine content. The phenylalanine is nitrated and subsequently reduced with hydroxylamine to give the intensely coloured purple salt.[132]

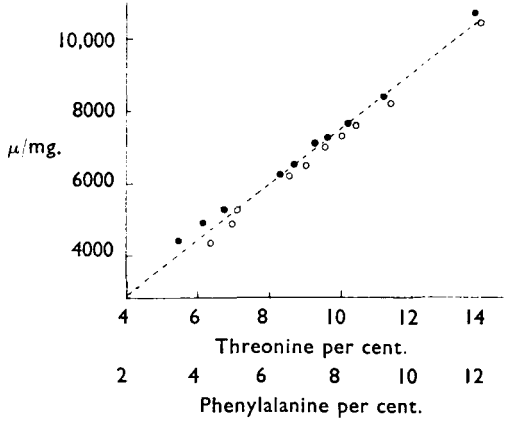


FIG. 1. Comparison of the two chemical methods for the assay of the polymyxins. Agreement between the two methods is good. Open circles, phenylalanine method; closed circles, threonine method.

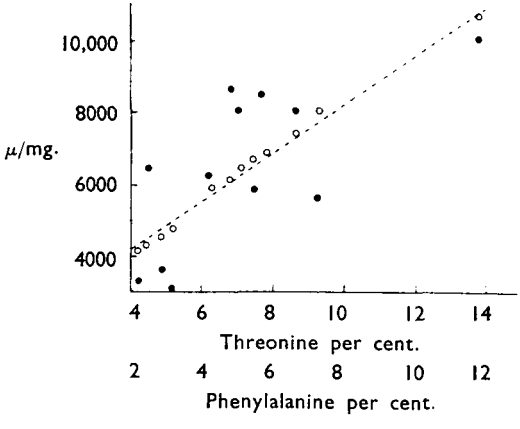


FIG. 2. Comparison of the microbiological and chemical methods for the assay of the polymyxins. The spread with the biological method is much greater than that obtained with the chemical methods. Open circles, chemical method; closed circles, biological method.

The 3 methods for the estimation of the polymyxins afford an interesting illustration of the relative accuracy of chemical and biological methods of assay. Figure 1 compares the accuracy of the two chemical methods and Figure 2 the biological method with the two chemical ones. The spread of the biological method is considerably greater than that shown by the two chemical methods.

One of the disadvantages of the chemical methods is that breakdown products containing the chemically reactive grouping would still be estimated although they might be biologically inactive. The close agreement between the two chemical methods, and the fact that the biological values are both higher and lower than those obtained chemically, indicate that in this case the difference between the relative accuracy of the biological and chemical methods is a true one.

Blood concentration time curves. The value of a quick and accurate method of estimation of a drug is that it enables absorption and excretion rates to be determined. Absorption curves are prepared by giving groups of animals varying doses of the drug, withdrawing blood at intervals and

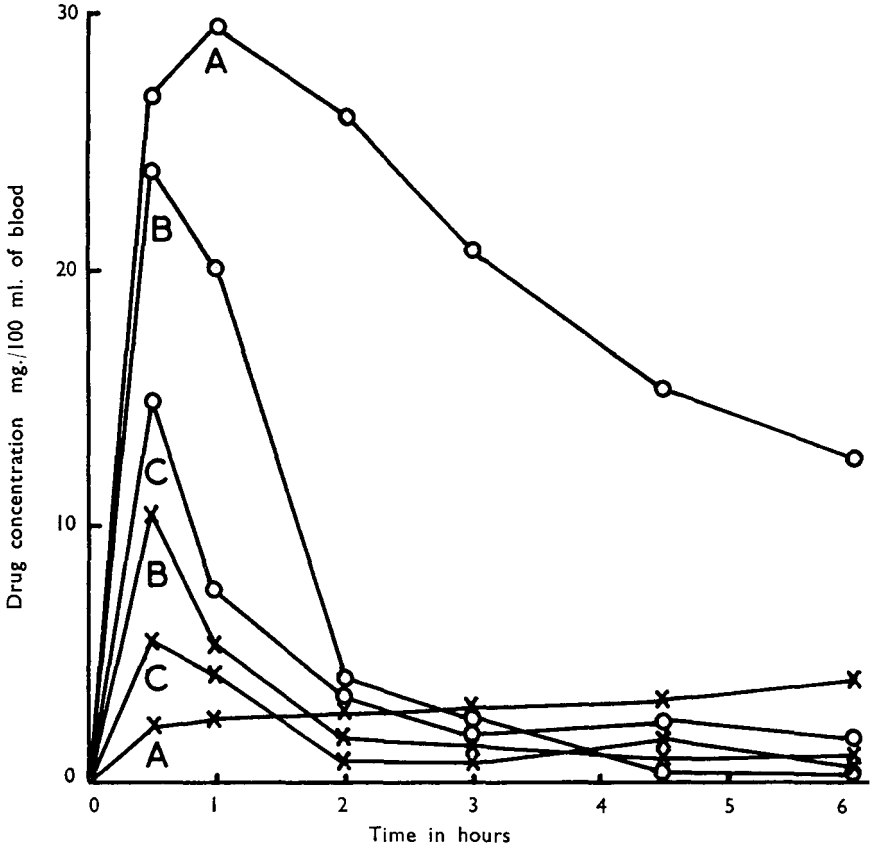


FIG. 3. Typical blood concentration time curves obtained after oral and parenteral administration of two drugs showing different types of absorption.

Sulphetrone ×———×
p-Aminosalicylic acid ○———○
A. Orally 625 mg./kg.
B. Subcutaneous injection 250 mg./kg.
C. Intraperitoneal injection 250 mg./kg.

determining its drug concentration. Absorption curves indicate the rate of clearance of drug from the blood and hence the frequency of dosage required. The ideal curve should reach a maximum rapidly, and the level should be maintained over a long period. Figure 3 shows typical blood concentration time curves with two drugs which show the two main types of absorption. *p*-Aminosalicylic acid is rapidly absorbed,

the maximum concentration being reached within half an hour and it is also rapidly cleared. Frequent dosage is necessary to maintain a therapeutic concentration in the blood. Sulphetrone is only slowly absorbed and the blood level remains fairly constant over several hours. Treatment once daily or less is sufficient to maintain a suitable blood level. Figure 3 also contrasts the blood levels produced after parenteral and oral administration. In general, higher blood levels are attained, the maximum

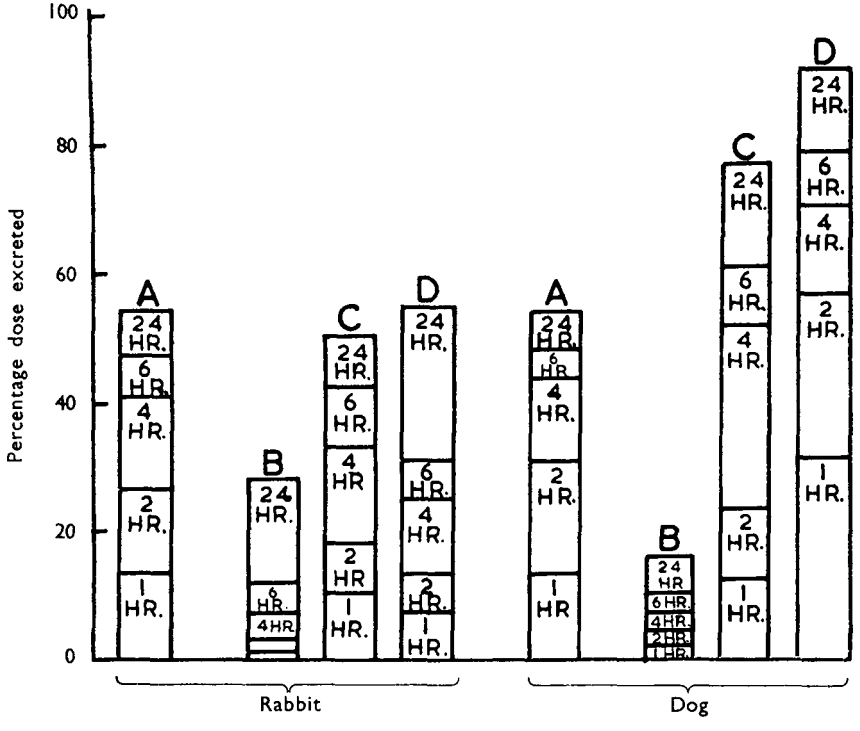


FIG. 4. Comparison of urinary excretion after administration of two drugs giving different types of absorption curves.

A. *p*-Aminosalicylic acid 200 mg./kg. administered orally
B. Sulphetrone 500 mg./kg. administered orally.
C. Sulphetrone 200 mg./kg. administered by intraperitoneal injection.
D. Sulphetrone 200 mg./kg. administered by subcutaneous injection.

concentration is reached more rapidly, and the drug disappears more quickly following parenteral administration than when it is given orally.

Excretion rates. The rate of urinary excretion may be determined similarly by administering the drug and determining the drug concentration in the urine at definite intervals of time. Correlation of excretion rates with absorption curves after parenteral administration is of value in determining the fate of the drug in the body; a rapid disappearance from the blood together with a high urinary excretion indicates that the

drug is being eliminated unchanged; a high blood level and a low excretion suggests that it is being stored in one or more organs, forming a depôt from which it is being absorbed, and a low blood level and a low excretion probably means that the drug is being broken down in the body.

Figure 4 gives the urinary excretion of *p*-aminosalicylic acid and sulphetrone in the rabbit and the dog after oral and parenteral administration. The high percentage excretion of *p*-aminosalicylic acid after oral and parenteral administration shows that the rapid fall in blood concentration is due to rapid urinary excretion and not to metabolism or storage of the drug. The difference in the excretion of sulphetrone after oral and parenteral dosage, shows that the relatively low blood level obtained after oral administration is due to poor absorption from the gut.

The absorption and excretion curves of penicillin are similar to those of *p*-aminosalicylic acid, about 80 per cent. appears in the urine in a few hours. Various attempts have been made to prolong the blood level, either by the administration of preparations which slow down the rate of absorption or by the simultaneous administration of substances, such as carinamide, which reduce the rate of urinary excretion.

Chronic Toxicity

Determination of the LD50 and therapeutic index give a measure of acute toxicity and ensures against the dangers of overdosage. A substance may, however, not be acutely toxic at the dosage employed, but may produce harmful effects with continual treatment over a period of time. Such effects have been reported with several drugs. Streptomycin, for example, has in some cases produced fever, an erythematous rash or neurological reactions with vestibular disturbances; continued dosage with acetanilide results in the formation of methæmoglobin and sulphone therapy tends to cause various side reactions such as nausea, dermatitis and depressive effects.

Indications of any harmful effects or histological damage likely to be produced by continued administration of the drug may be obtained by chronic toxicity tests. Chronic toxicity tests are performed on several species, to eliminate any special sensitivity or resistance in any one species. The animals are treated with 3 to 5 times the therapeutic dose continually for periods of a few weeks to several months, depending on the dosage scheme envisaged for the drug during therapeutic use.

General observations made to detect any abnormal effects include daily records of temperature and blood pressure and the effect on body weight. The value of weight variation as an indication of toxic effects is shown in the results recorded in Figure 5 of a growth rate test with the polymyxins.

In addition, a complete hæmatological examination, a thorough qualitative and microscopical examination of the urine for abnormal constituents and a quantitative estimation of the main constituents of blood are made at frequent intervals. These tests all help to indicate various impairments of function. Changes in the hæmatopoietic system

are reflected in the hæmatological picture, increased blood sugar and blood urea concentration indicate impaired renal function and excretion of various abnormal constituents suggest the possibility of kidney or liver damage.

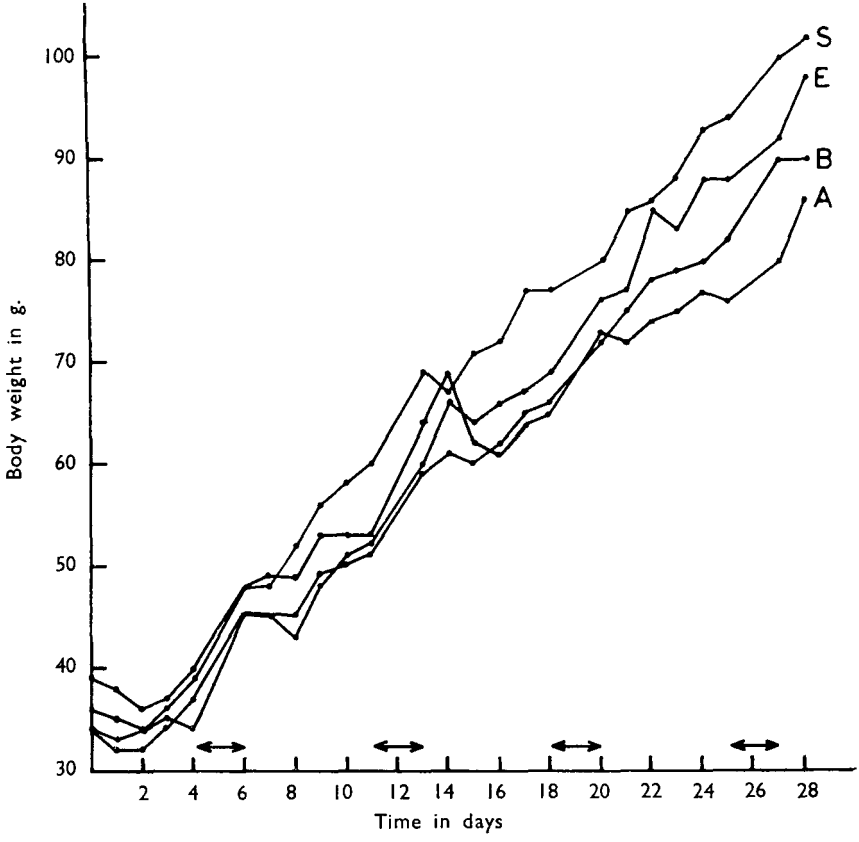


FIG. 5. Growth rate test with the polymyxins in young rats. Groups of young rats, two to three weeks old were treated three times daily with 5000 units of the polymyxins. Treatment was continuous except at the arrows.

A. Polymixin "A."
B. Polymixin "B."
E. Polymixin "E."
S. Saline.

Kidney damage is usually accompanied by proteinuria. 3 of the 5 members of the polymyxin group of antibiotics were found to show a reversible nephrotoxicity in animals and man. This was readily detected by a marked proteinuria. Table III shows the increased protein excretion produced by polymyxin A in the rat, rabbit and dog, and illustrates the value of using several different species in these tests. The rat is not the animal of choice for the study of kidney function because a high proteinuria is usually found in the normal animal. Rabbits are not very sensitive

to nephrotoxic substances and some proteinuria is frequently found in untreated animals. The test is critical in the dog, since normal animals may be selected showing no initial proteinuria and the dog is extremely sensitive to nephrotoxic compounds.

TABLE III

PROTEINURIA IN ANIMALS AFTER ADMINISTRATION OF POLYMYXIN A

Animal	Dose	Weight protein in mg. excreted in					
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Rat	10,000 U./100 g.	15	75	58	34		
		12	47	75	31		
		18	79	60	33		
	Control	14	12	13	13		
Rabbit	30,000 U./kg. for 5 days	9	40	185	78		
		0	0	310	260	26	38
	Control	11	3	1	16	2	3
		Concentration of protein (g./l.) in urine					
Dog	10,000 U./kg. 4 times daily for 3 days	0	0·1	3·7	1·0	0·5	0·3
		0	0·3	2·5	1·4	1·0	1·0
		0	0·1	1·4	4·4	0·5	0·7

At the end of the period selected for the chronic toxicity tests, half the animals are killed and the tissues examined histologically to confirm and extend any clinical findings. The remainder are kept for a few weeks without further treatment before being killed, in order to determine whether any damage which may have been produced is permanent or reversible.

TABLE IV

EXISTING BACTERIAL CHEMOTHERAPY

Organism	Penicillin	Sulphonamides	Streptomycin	Aureomycin	Chloramphenicol	Terramycin
Staphylococcus aureus	++	+/+	+	+	+	+/+
Streptococcus pyogenes	++	+/+	+/+	+	+	+/+
Streptococcus agalactiæ	+/+	+/+	+	+/+	+	+/+
Streptococcus pneumoniæ	++	++	+	+/+		?
Neisseria gonorrhœæ	+/+	++	+/+	+	+/+	?
Neisseria meningitidis	++	++	—/+	+/+	+	?
Streptococcus viridans	++	—	—	+/+	+	
Clostridium welchii	++	—	—			
Corynebacterium diphtheriæ ..	++	—	—			
Actinomyces bovis	++	—	—			
Treponema pallida	++	—	—	+	+	+
Bacillus anthracis	+/+	—	—			
Mycobacterium tuberculosis ..	—	—	+/+	—	—	—
Escherichia coli	—	—	+	++	++	+
Hæmophilus influenzæ	—	—	—	++	+	+
Hæmophilus pertussis	—	—	—	+	++	?
Salmonella typhi	—	—	—	+	++	
Salmonella infantis	—	—	—	+	++	
Shigella	—	—	—	++	++	
Brucella	—	—	—	++	++	?
Pseudomonas pyocyanea	—	—	+/+	—	—	?
Proteus	—	—	+	+	+	?
Klebsiella pneumoniæ	—	—	—	++	+	++
Rickettsia	—	—	—	++	++	?
Lymphogranuloma venerelum ..	—	—	—	++	++	
Psittacosis	—	—	—	++	++	
Other viruses	—	—	—	—	—	—

++ Most satisfactory treatment. + Active. ? Preliminary reports satisfactory. — No effect.

The Present Position

Progress in chemotherapeutic control, especially of bacterial infections, has made phenomenal advances during the last 15 years, and the systematic preparation and testing of potentially active compounds has contributed considerably to this end. Most of the diseases due to Gram-positive pathogens can now be more or less adequately controlled by penicillin, the sulphonamides and streptomycin, and with the introduction of the polymyxins, aureomycin, chloramphenicol and terramycin, equally good results are being obtained with many of the Gram-negative and some of the Rickettsial infections. Table IV summarises the present position in the chemotherapy of bacterial diseases. The three "wide-spectrum" antibiotics, aureomycin, chloramphenicol and terramycin are not, as at first hoped, active against true small viruses, and there thus remains a wide field of research which is still comparatively untouched.

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