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THE CHEMOTHERAPY OF AMOEBIASIS

PART I. INTRODUCTION AND METHODS OF BIOLOGICAL ASSAY

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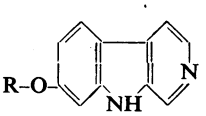
Three types of drug are commonly used for the treatment of amoebiasis: (*a*) emetine or its sparingly soluble derivatives such as the bismuth iodide (" E.B.I.") ; (*b*) halogenated quinolines such as 7-iodo-8-hydroxyquinoline-5-sulphonic acid (chiniofon B.P., " yatren," " quinoxyl "), 5-chloro-7-iodo-8-hydroxyquinoline (" vioform ") and 5 : 7-diiodoquinoline (" diodoquin ") ; and (*c*) arsenical preparations such as 4-carbamidophenylarsonic acid (carbarsone) and 3-acetamido-4-hydroxyphenylarsonic acid (acetarsone B.P.C., " stovarsol "). It is usual in current practice to administer courses of these drugs together with retention enemata of chiniofon. Recently penicillin and sulphonamides have also been given to control secondary infections and this treatment has been successful in a number of cases previously resistant to treatment with amoebicides alone (Hargreaves, 1945a and b ; 1946).

In spite of this armoury of medicaments there is still a great need for a new drug, not only to deal with refractory cases, but also if possible to avoid the objectionable properties of emetine. Ipecacuanha is scarce and expensive, and its most important alkaloid, emetine, although highly specific, is toxic and even in its pharmaceutically most elegant forms causes nausea and vomiting in many patients. The systematic search for new amoebicides has been hampered by the lack of a reliable *in vitro* test and of a convenient experimental infection in animals.

Pyman (1937a and b) examined a homologous series of harmol ethers (I) in which R was an alkyl group containing from two to twelve C atoms. A peak in amoebicidal activity



(I)

$(C_5H_{11})_2N–(CH_2)_{10}–N–(C_5H_{11})_2$

(II)

in vitro was found with $R=C_9H_{19}$ but the compounds were very sparingly soluble. In order to increase the solubility, basic groups were introduced into the terminal position of the alkyl groups [(I),$R=Alk_2N(CH_2)_x$] ; the number of methylene groups and the size of the alkyl groups were varied and a peak in activity was found in O-11-di-*n*-butylamino-undecylharmol [(I),$R=(C_4H_9)_2N(CH_2)_{11}$-] which, under the conditions of the test used, was lethal to *Entamoeba histolytica* in a dilution of 1 : 750,000 to 1 : 4,000,000. As this was many times more active than O-*n*-nonylharmol it was conjectured that the harmol residue might not play an important part in the amoebicidal action, and this part of the molecule was replaced by other groups, leading ultimately to the preparation of 1 : 10-bis-(di-*n*-amylamino)-decane (II) which was found to be three to five times as efficient as emetine. Unfortunately a clinical trial failed to confirm the promise of the *in vitro* test. It is evident from this careful work of Pyman and his collaborators that an *in vitro* test alone is not enough to indicate whether or not a compound is likely to be useful in the treatment of amoebiasis.

The present series of papers describes an attempt to synthesize amoebicidal substances based upon the structure of emetine, and upon the structure of certain compounds found by Pyman to be active *in vitro*. Details of the methods used for biological tests, and the results obtained with standard amoebicides will be described in the present paper, and an account of the new compounds prepared and their biological activities will be given in Parts II and III.

IN VITRO AMOEBICIDAL TESTS

The activity of ipecacuanha alkaloids upon free-living amoebae was demonstrated by Vedder (1911) and by Pyman and Wenyon (1917), but satisfactory evidence of specific activity against *E. histolytica* was not forthcoming until a medium was devised which would maintain the amoebae alive sufficiently long for the drugs to take effect, and which contained no solid phase to remove the alkaloid

from solution. The buffered horse-serum Ringer medium (hs) of Laidlaw, Dobell, and Bishop (1928) met these requirements and was used by Pyman and his collaborators (1937a and b) for the examination of new compounds. A further source of difficulty has been the fact that so far it has not been possible to grow *E. histolytica* in the absence of bacteria, and the results of *in vitro* tests have been complicated by the presence of an indeterminate mixed bacterial flora used by the amoeba as a source of essential metabolites. Recent American work (Rees, Reardon, Jacobs, and Jones, 1941) has shown that it is possible to grow the organism in the presence of single strains of bacteria. In this country Dobell has succeeded in growing *E. histolytica in vitro* in the presence of a single pure strain of *Bact. coli*, and it was reported by Hargreaves (1945) that in such a culture the amoebae were killed by a concentration of emetine hydrochloride as low as 1 : 5,000,000. The use of this culture enables the observer to detect bactericidal action of a new compound which might be confused with direct amoebicidal action in a culture containing many strains of bacteria, some but not all of which were affected by the drug. The work of Pyman and the more recent work of Rawson and Hitchcock (1947) suffer from the disadvantages resulting from the presence of a mixed bacterial flora.

Methods used in the present work

For the cultures used, and for the method of test described below, we are indebted to Dr. Clifford Dobell. Two strains of amoeba have been used, both growing in culture with a single "smooth" strain of *Bact. coli.* One of them was obtained from a natural infection in a monkey, and was morphologically indistinguishable from *E. histolytica*; the other was isolated from man. These strains were identical in their reaction to emetine *in vitro.* The cultures were maintained on the horse-serum Ringer-egg (HSre) medium of Dobell and Laidlaw (1926). For an *in vitro* test, a series of 4 in.×1/2 in. tubes were prepared, each containing 4.5 ml. of buffered horse-serum—Ringer medium (hs) and 0.5 ml. of a sterile solution of drug in buffered Ringer. Each was inoculated with 6 drops (0.2 ml.) of a heavy culture of *Bact. coli* in Douglas-Hartley broth, and a large loopful (20 mg.) of dry, sterile rice starch added. The tubes were incubated overnight at 37° C. for growth of the bacteria to take place, and for the conditions at the base of each tube to become anaerobic. This may be confirmed in a sample tube by the addition of methylene blue before incubation; if growth is satisfactory the dye is bleached at the bottom of the tube. A heavy suspension of *E. histolytica* was then added to each tube, the inoculum being placed carefully on to the surface of the starch with a pipette. Large quantities of inoculum were obtained by culture in flasks of HSre medium prepared by the method of Frye and Meleney (1935). After incubation for three days, the tubes were examined microscopically for the growth of amoebae, the bacteria present tested for viability by subculture, and the *p*H of each tube of culture checked. The results obtained with standard amoebicides are recorded in Table I.

TABLE I

THE ACTIVITY OF AMOEBICIDAL DRUGS *in vitro*

Drug	Growth of amoebae in the presence of various concentrations of drug			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Emetine HCl ..	−	−	±	+
Carbarsone ..	±	+	+	+
Acetarsone ..	+	+	+	+
Chiniofon ..	−	+	+	+
Diodoquin* ..	±	+	+	+

*Almost insoluble; solid particles present.

None of these drugs had bactericidal activity at the dilutions used.

In Vivo Amoebicidal Tests

Still greater difficulties have been encountered in the finding of a suitable experimental infection upon which the *in vivo* activity of drugs could be investigated. Thousands of cats have been used in some laboratories (Wagner, 1935; Bieling, 1935; Kikuth, 1945) in spite of the fact that Dale and Dobell had shown in 1917 that the acute and lethal infection produced in kittens by the inoculation of *E. histolytica* per rectum was not affected by doses of emetine large enough to be toxic to the host, and that therefore experiments with new drugs could not be expected to yield much useful information.

Several workers have reported infection of rats and mice by the oral inoculation of *E. histolytica* cysts but such experiments have not been uniformly successful in the past. Meleney and Frye (1932) and Deschiens and Provost (1937) described the infection of cats by the direct inoculation of trophozoites into the lower ileum: in a recent paper by Jones (1946) this method has been modified and applied successfully to young rats, and a method of testing drugs has been carefully worked out. Our own experiments made independently during the past two years and described in the present series of papers produced similar results, showing that the young rat is a suitable host for caecal infections of *E. histolytica.* The infection in the rat caecum differs from that in the human

gut in that we have never yet seen an ulcer of the deep flask-shaped type in which amoebae are living exclusively upon the tissues of the host. The ulcers are extensive but shallow and rarely involve tissues deeper than the mucosa. The active amoebae frequently contain bacteria, whereas ingestion of red cells is only occasionally encountered, and cysts are rare. Nevertheless, as infected rats can be cured by the drugs used in human therapeutics, we consider the method to be the best so far devised for the investigation of new compounds designed as amoebicides.

Methods used in the present work

Animals

Very young rats weighing 20–30 g. were used immediately after weaning. At this age the animals have not developed an extensive caecal flora and fauna, having had milk as their main diet. The caecum is small and almost empty apart from a mucous secretion. At a later age the caecum becomes enlarged and distended with food residues and living organisms, and the inoculation of *E. histolytica* is less likely to produce an acute infection. Chronic light infections could be produced in older rats, but were less suitable for therapeutic experiments.

We were troubled in the early stages of this work by natural infections of the rats by an amoeba resembling *E. muris*. This organism is distinguishable from *E. histolytica* in stained smears, but is difficult to detect in fresh preparations especially when *E. histolytica* is also likely to be present. We also had the impression that a rat infected with *E. muris* was difficult to infect with *E. histolytica*, caecal conditions favourable to the former being apparently unsuitable for the latter. Experiments in these laboratories by Neal (1947) showed that hamsters frequently harboured heavy caecal infections of *E. muris* and discharged cysts which were infective to rats. They therefore served as a reservoir from which young rats readily became infected. Rats could be kept free from infection by keeping them in a room apart from hamsters and by bedding them in clean wood-wool sterilized by heating at 150° C. for 1 hour.

Strain of amoeba

We have tried a number of strains isolated from human patients, and find them to be variable in their pathogenicity to rats. It is possible that this is caused by differences in associated bacterial flora. The strain we have used for routine tests ("Strain B") was derived from a man infected in Burma, and had resisted a total of 11 courses of treatment with emetine and other drugs before it reached our hands. We are indebted to Air Commodore T. C. Morton for the material. This organism when grown in bulk upon HSre medium and inoculated intracaecally caused infection of 80 to 100 per cent of young rats. We did not find it necessary to add mucin to the inoculum as recommended by Jones (1946).

Method of inoculation

The rats were lightly anaesthetized with ether, the skin of the abdomen shaved, and the caecum exposed through a very small incision slightly to the left of the mid-line. A volume of 0.2 to 0.4 ml. of a rich suspension of amoebae from culture was injected directly into the caecum, the jet of fluid being directed in such a way that the blind end of the caecum was completely filled and good contact was made with the mucosa. A small amount of a mixture of equal parts of sulphanilamide and marfanil (about 10 mg.) was dusted into the abdominal wound to minimize chances of secondary infection, and the wound was closed with a rayon suture in the muscle and a Michel clip in the skin. The dusting with sulphonamides has no effect upon the development of the amoebic infection.

Treatment with drug

The rats were divided into groups and fed for six days upon diet containing the drug to be tested. The diet was a Coward stock diet, and the amount of drug incorporated was determined by the results of toxicity tests. The daily food consumption of a newly weaned rat is about 4 g. per day and the maximum concentration used for any drug was 0.5 per cent, representing a daily dose of about 800 mg. per kg. A disadvantage of the drug–diet technique is that the drug may be distasteful to the rats, and the food intake is then less than normal. This can readily be detected by observation of the food consumed and if a new drug shows promise as an amoebicide, more accurate treatment can be ensured by frequent dosage by catheter. The drug-diet method is convenient for "screening" tests and ensures continuous treatment with drugs. A group of untreated infected controls was used in every test to check the infectivity of the inoculum.

Assessment of results

For the assessment of the effect of a drug, the criterion we used was the presence or absence of amoebae in the caecum, as demonstrated by microscopical examination and by culture in HSre medium. Caecal contents showing plentiful amoebae under the microscope will not always grow out in culture if the concomitant bacterial flora is unsuitable, but culture will sometimes demonstrate the presence of an infection not detected microscopically, and is thus a useful supplement to direct examination. The condition of the caecum of each rat was also assessed by a "scoring" method similar to that described by Jones (1946). The presence of amoebae did not enter into our scores, which were awarded upon macroscopical evidence only. The conditions of the caecal wall (normal=0; extensive ulceration=4) was scored separately from the condition of the contents

(normal = 0; mucus only = 4). We have found that a drug may provide marked protection of the caecal wall and yet not remove the amoebae from the contents; another drug may remove the amoebae, but as a result of its irritant action the caecal wall will appear thickened and the contents fluid or mucoid. Such examples cannot be scored by Jones's method, and we consider that the alternative system described above gives a better analysis of the effects of a drug.

The results obtained with a series of standard amoebicides are shown in Table II.

TABLE II

THE ACTIVITY OF AMOEBICIDAL DRUGS *in vivo*

Drug	Concentration in diet %	Condition of caecum		Rats free from infection	
		Wall (mean score)	Contents (mean score)	Proportion	Per cent
Normal rats ..	—	0	0	—	—
Infected controls	—	3.0	2.8	30/156	19
Emetine HCl ..	0.002	0.2	1.0	16/18	89
	0.001	0.6	0.5	12/15	80
	0.0005	1.3	0.9	2/8	25
	0.00025	1.3	1.3	0/4	0
Emetine bismuth iodide	0.05	1.9	1.7	9/9	100
	0.02	1.5	2.8	26/29	90
	0.01	1.0	1.7	27/30	90
	0.005	1.7	1.9	15/17	88
	0.002	2.0	2.1	1/7	14
Carbarsone ..	0.4	0	0.3	6/6	100
	0.2	1.6	1.7	10/22	45
	0.1	2.1	2.1	3/8	38
Acetarsone ..	0.4	0	0.6	7/7	100
	0.2	1.0	1.2	12/23	52
	0.1	1.3	1.1	3/8	38
Chiniofon ..	0.2	0	0	8/8	100
	0.1	2.8	2.5	4/17	24
	0.05	3.6	2.6	0/7	0
Diodoquin ..	0.5	0	0	8/8	100
	0.2	3.0	2.9	4/12	33
	0.1	2.9	3.0	2/9	22

Discussion

The activities of drugs *in vitro* shown in Table I demonstrate the highly specific action of emetine. The limiting amoebicidal concentration of 10^{-6} to 10^{-7} is in agreement with the findings of Dobell (reported by Hargreaves, 1945; 1946). All the other drugs were much less active, or showed no activity at 10^{-4}. The results of the *in vivo* tests (Table II) also show emetine to be the most active drug tested in spite of the fact that the strain was derived from a case which had resisted repeated courses of this and other drugs. Emetine bismuth iodide showed activity commensurate with its alkaloidal content; the inorganic fraction appeared to have no influence upon the potency of the drug in the acute experimental infection. The results of giving emetine in the diet were more successful than those reported by Jones (1946) in which subcutaneous injection of six doses of 2 mg./kg., or oral administration of a single dose of 12 or 15 mg./kg. protected only 60 per cent or less of the animals.* A drug diet containing 0.001 per cent of emetine HCl, corresponding to a daily intake of approximately 1.5 mg./kg. was sufficient to protect 12/15 rats infected with strain "B," though we have evidence that strains derived from other sources may be less susceptible to emetine. Rats receiving drug diet containing 0.02 or 0.05 per cent of E.B.I. showed considerable irritation of the caeca, the walls appearing thickened and pale and the contents fluid. This is shown by the higher scores at these dose levels.

Comparison of Tables I and II shows that the drugs tested fall into the same order of relative activity by both *in vitro* and *in vivo* methods. Experiments with other drugs, which will be recorded in Parts II and III, have shown that this is not always the case. In the past it was a common practice to calculate the "chemotherapeutic index" of a drug from the ratio:

$$\frac{\text{"maximum tolerated dose"}}{\text{"minimum curative dose"}}$$

These terms have fallen into disfavour because their errors are large and indefinable and an attempt to provide a more accurate index has been made by expressing it as the ratio of the median lethal and median effective doses (Wien, 1946). Dr. J. W. Trevan, at the January, 1947, meeting of the British Pharmacological Society, pointed out that such a ratio was of little value if the dose–response curves were not parallel, and also that it did not express the relationship required—namely, a comparison of the safe dose with the curative dose. He suggested that a better figure would be given by the ratio of the "LD 0.1" and the "CD 99.9," which can be regarded as accurate substitutes for "maximum tolerated" and "minimum curative" doses. These figures may be determined from the dose–response curves by extrapolation or by calculation from the regression formulae.

In Table III this method has been applied to the amoebicides. Toxicity determinations were made upon rats of the same age and strain as those

* *Added in proof.* In a more recent paper Jones (1947) reports results from repeated oral administration of drugs which are very similar to those found by us.

used for amoebicidal tests, and the figures were obtained graphically. The total daily dose commonly employed in human therapeutics is also recorded, and will be seen to compare reasonably well with the daily intake required to cure rats.

TABLE III

Drug	Maximum clinical daily dose mg./kg.	Acute toxicity to young rats LD 0.1 mg./kg. orally	Amoebicidal activity CD 99.9 mg./kg./day in diet	Chemotherapeutic index $\frac{LD\ 0.1}{CD\ 99.9}$
Emetine HCl ..	1	6	4.5	1.3
Emetine bismuth iodide ..	3	11	15	0.73
Carbarsone ..	14	4,500	600	7.5
Acetarsone ..	10	1,200	600	2
Chiniofon ..	30	800	300	2.7
Diodoquin ..	30	>40,000	600	>67

Table II shows that, in spite of their specific activity, it was difficult to obtain 100 per cent of cures with emetine HCl or emetine bismuth iodide. This was probably because emetine drug-diets containing more than about 0.001 per cent are irritant and distasteful to rats; the total dose therefore may not have been taken each day. On the other hand individual rats may carry infections which resist treatment with the alkaloid. In either event the question requires further investigation. The CD 99.9 values for these two drugs were estimated by extrapolation of the linear parts of the dose–response curves and are therefore probably too low. Reference to Table III shows that even these low figures are for emetine bismuth iodide within, and for emetine hydrochloride almost within, the toxic range. Chiniofon is the next most active, and also the next most toxic drug. Carbarsone appears to be better than acetarsone because of its lower toxicity, and diodoquin has the highest chemotherapeutic index of all because no rats died after an oral dose as high as 40 g./kg. Since it has no appreciable activity *in vitro* even when solid particles of drug are present (Table I), it is probable that diodoquin is converted to a more active substance during its passage through the body.

In clinical practice none of the drugs is successful in every case.

Summary

1. Methods are described for the comparison of the amoebicidal activities of drugs: (*a*) *in vitro*, using a strain of *Entamoeba histolytica* growing in the presence of a single strain of *Bacterium coli*; and (*b*) *in vivo*, using young rats inoculated intracaecally with cultures of amoebae.

2. The following standard amoebicides have been tested: emetine, emetine bismuth iodide, carbarsone, acetarsone, chiniofon, and diodoquin. Emetine was the most active, both *in vitro* and *in vivo*.

3. The chemotherapeutic indices of the drugs have been calculated and show that the curative dose of emetine is very close to the toxic dose. Of the drugs tested, diodoquin had the most favourable chemotherapeutic index.

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