Brit. J. Pharmacol. (1960), **15**, 436.

SCREENING OF CESTICIDAL COMPOUNDS ON A TAPEWORM *HYMENOLEPIS NANA IN VITRO*

BY

A. B. SEN AND F. HAWKING

From the Central Drug Research Institute, Lucknow, India

(RECEIVED MARCH 30, 1960)

A simple and convenient *in vitro* technique is described for the screening of compounds for action against *Hymenolepis nana* and probably many other intestinal worms. The results obtained from this test are in broad agreement with the findings of clinical experience and of a small series of *in vivo* tests. Among the substances tested, the most active ones were oil of chenopodium, dichlorophen, extract of cashew nut (*Anacardium occidentale*), antimony potassium tartrate, and BIQ 20 [eicosamethylenebis(isoquinolinium iodide)].

The testing of possible chemotherapeutic compounds upon helmintic infections in general is usually a cumbersome process which consumes great amounts of the chemical specimen, of animals and of time. In the present paper an *in vitro* technique is described for testing compounds on intestinal worms such as *Hymenolepis nana*; this technique is simple, and economical of time, animals and the specimen under test. It could be applied to many other intestinal worms. A preliminary notice of this work has been given elsewhere (Sen and Hawking, 1959).

METHODS

The worm selected for this work was *Hymenolepis nana,* which is a common tapeworm of mice, and which also occurs in man, especially in the Mediterranean area, Near East, and S. America; it can be transmitted directly by ova from mouse to mouse, since both stages of the worm (cysticercoid and tapeworm) occur in the same host. The worm was maintained in mice according to the technique described by Steward (1955). Ova were obtained from ripe segments of worms taken from infected mice. It was necessary to check under the microscope that the ova were mature since immature eggs will not infect mice. The eggs were freed from the segments of worms by light crushing under a cover slip, and they were then washed into boiled and cooled tap water. They were allowed to stand in water for 20 (or 44) hr. at room temperature (about 30°). The ova were then counted in a haemocytometer chamber, and an appropriate number (500 to 2,000) were given to mice by syringe and stomach tube.

Twenty to forty days after inoculation the mice were killed, having been starved for 24 hr. beforehand. The intestine was washed out, by inserting the needle of a syringe into the upper end and perfusing it with saline. If an isotonic solution buffered to be slightly acid (*p*H 6.8) was employed, the worms came out more readily. Alternatively, the small intestine freed from the mesentery was placed between two thick glass plates which were clamped together and examined by the naked eye or a binocular dissecting microscope. Worms *in situ* could be seen well by this technique, but they were difficult to recover from the intestine subsequently by perfusion. Newly weaned mice seem more susceptible than other mice. Some of the mice bred at this Institute contain natural infections of *H. nana* so that some older mice may possess an immunity from previous infections and some of the young mice may possess a partial immunity transmitted by immune mothers. Under good conditions the percentage of mice which later contained worms was about 80% and the number of worms was from 1 to 56 in each mouse. If only one worm was present 21 days after infection, it was usually a large one about 5 cm. long. If many worms were present they were small, from 0.5 to 1 cm. In this Institute at one season of the year, laboratory animals are often given cucumber as green food. There is some evidence that this had an anthelmintic action. Thus, of 7 control mice fed on stock diet, 6 (86%) were infected and they contained 3 to 13 worms each; while out of 5 mice fed with cucumber, only 2 were infected and they contained 3 and 5 small worms respectively. Accordingly, it is better to avoid a cucumber diet. A diet containing much potato seems to be favourable.

To expose the worms to compounds *in vitro,* the worms recovered from the intestine by perfusion were picked up free from mucus and other debris and transferred to two washes of Ringer solution. They were then placed in nutrient broth containing penicillin 500 units per ml. and streptomycin 0.1 mg. per ml.

The formula of the nutrient broth was as follows :

Peptone	10 g.
Yeast extract	2.5 g.
Sodium chloride	5 g.
Distilled water	1,000 ml.

The *p*H was adjusted to 8.5 by adding 2 ml. of 40% sodium hydroxide solution. After 0.5 to 1 hr. in nutrient broth at room temperature (30 to 34°) they were transferred to 3 ml. of the same medium in a suitable container. For this purpose small Kjeldahl's flasks of 10 ml. volume were used. Concentrations of penicillin as high as 2,000 units per ml. and of streptomycin as high as 1.0 mg./ml. were harmless to the worms. One or two worms were placed in each flask, which was then bunged. Suitable concentrations of the various compounds had been previously added to the nutrient broth in each flask. Since it was desired to make a broad survey of a large number of compounds, concentrations were chosen in multiples of 10. If the compound was insoluble in water, it was ground up, and a fine suspension was made by shaking in broth; sometimes the compound was dissolved in alcohol before adding to broth. In these cases the maximum concentration of the compound depended on its solubility in the broth, and the concentrations recorded in the table are nominal ones, the actual concentration being lower. Preliminary experiments showed that concentrations of alcohol greater than 1% are harmful to the worms and such concentrations were avoided. The flasks were incubated at 37°. For examination they were placed in a horizontal position under a binocular dissecting microscope with low magnification where the movements of the worms could easily be observed. The worms of the control flasks with drug-free medium remained actively mobile for 5 days. The action of the drug, however, was read after 24 hr. because it is unlikely that a drug given by mouth would stay in the intestine even as long as 24 hr. Bacterial growth in the flask did not occur to any appreciable extent.

Some tests were made, for comparison, of the action of the drugs *in vivo*, using the technique of Steward (1955). Groups of mice were taken on the 14th day after infection. The drug was given in a single dose by oral catheter. The mice were starved on the second day and killed on the third day after treatment. The worms were collected from the intestine and scored by the number of worms present multiplied by a factor for the size of the worm (worms more than 3 cm., 20; about 2.5 cm., 10; about 1.2 cm., 5; about 0.6 cm., 0.5; less than 0.3 cm., 0.1). The results thus obtained were compared with those from a control group of untreated mice.

Results

The results obtained during the *in vitro* experiments are shown in Table I; each figure is based on five separate experiments. The readings with carbon tetrachloride and tetrachlorethylene were very variable, ranging from 1:1,000 to

Table I

THE MINIMUM CONCENTRATIONS OF VARIOUS COMPOUNDS REQUIRED TO KILL *HYMENOLEPIS NANA* AT 37° IN 24 HR.

The extracts dichlorophen and oil of chenopodium were dissolved in alcohol and then diluted with water and Ringer solution. The solubility was greater than the minimum lethal concentration except for sulphadiazine (soluble 1:1,300), santonin (very slightly soluble), tetrachlorethylene, phenothiazine, pamaquin (insoluble), carbon tetrachloride (soluble 1:2,000).

Compound	Minimum Lethal Concentration
Male fern extract	1:100,000
Extract of cashew nut (ether extract of *Anacardium occidentale*)	1:1,000,000
Oil of chenopodium	1:10,000,000
Thymol	1:10,000
β-Naphthol	1:10,000
Arecoline	1:100,000
Santonin	1:1,000
Hexylresorcinol	1:100,000
Dichlorophen (Dicestal)	1:2,000,000
Carbon tetrachloride	1:10,000
Tetrachlorethylene	1:10,000
Phenothiazine	1:1,000
Mepacrine hydrochloride	1:100,000
Acriflavine	1:100,000
Chloroquine phosphate	1:100,000
Pentaquine phosphate	1:100,000
Pamaquin	1:100,000
Stilbamidine isethionate	1:1,000
Propamidine isethionate	1:1,000
Piperazine	1:1,000
Diethylcarbamazine	No action
Arsenamide [(*p*-carbamoylphenylarsinidenedithio)diacetic acid]	1:10,000
Melarsoprol (Mel B)	1:1,000
Antimony potassium tartrate (tartar emetic)	1:1,000,000
Copper sulphate	1:1,000
Sulphadiazine	No action
Iodochlorhydroxyquin (Vioform)	1:100,000
Bialamicol hydrochloride (camoform)	1:1,000
Lucanthone hydrochloride	1:10,000
Quinapyramine (Antrycide)	1:1,000
Prothidium (7-amino-2-(2-amino-6-methylpyrimidin-4-ylamino)-9-*p*-aminophenylphenanthridine 10,1'-dimethobromide	1:1,000
BIQ 20 [eicosamethylenebis(isoquinolinium iodide)]	1:1,000,000
Penicillin (1,000 units per ml.)	No action
Streptomycin (1 mg. per ml.)	,, ,,
Chloramphenicol ,,	,, ,,
Tetracycline ,,	,, ,,
Ethyl alcohol	1:50

1 : 100,000 in 11 experiments ; these results may be related to the insolubility of these compounds in watery solutions, although preliminary solution in alcohol or acetone did not make the results any more consistent.

The compounds tested can be divided into groups according to their ability to kill the worms *in vitro* in 24 hr. The most active compounds, which kill at a concentration of 1/million or less include oil of chenopodium (1 : 10 million), extract of cashew nut (*Anacardium occidentale*), dichlorophen, antimony potassium tartrate (tartar emetic) and BIQ 20 [eicosamethylenebis(isoquinolinium)]. The moderately active compounds (killing at 1/100,000 concentration) include male fern extract, arecoline, hexylresorcinol, mepacrine, acriflavine, chloroquine, pentaquine, pamaquine, and iodochlorhydroxyquin (Vioform). The other compounds tested are relatively or absolutely inactive against *Hymenolepis*.

A few experiments were carried out in mice to compare the *in vivo* action of several well-known anthelmintics with the findings obtained *in vitro*. The results are shown in Table II. The compounds which were active *in vivo* had already shown activity *in vitro* with the exception of tetrachlorethylene (which had given very irregular results *in vitro*).

TABLE II

THE ACTION OF CERTAIN DRUGS UPON *HYMENOLEPIS NANA IN VIVO*

Compound	Dose mg./kg.	No. of Mice Free of Worms / No. of Mice Treated	Average Score	Evaluation of Treatment
Male fern extract	750	4/5	1·1	Active
Arecoline	20	3/5	3·2	,,
Hexylresorcinol	500	4/5	1·7	,,
Dichlorophen	100	5/5	0	Very active
Tetrachlorethylene	1·5	3/5	4·2	Active
Mepacrine	300	4/5	0·4	Very active
Untreated controls	—	0/5	32·2	

DISCUSSION

The chemotherapy of helminthiasis has been surveyed in excellent reviews by Watkins (1958) and by Whitten (1956). Accordingly the present discussion will be limited to brief comments on certain aspects.

Technique of Testing.—Many techniques for testing anthelmintics have been described, but methods involving the survival or death of tapeworms *in vitro* have not been used. The method described above is simple and economical ; the worms from a few mice are sufficient to test many different drug-concentration combinations ; only a minute amount of chemical compound is required ; no previous toxicity tests are required. Theoretically, an *in vitro* method seems justified for screening compounds upon intestinal worms (although not upon parasites in the blood or tissues) since intestinal worms live in the lumen of the gut and anthelmintic drugs which have been given by mouth reach the parasites in the intestine without much opportunity for chemical modification. Of course, no single chemotherapeutic test can be guaranteed to detect 100% of active compounds, but as a compromise between expense and efficiency the present test seems good. The method is applicable to other parasites of the intestine and it is planned to apply it to some of the nematode worms. Preliminary experiments have shown that *Nippostrongylus muris* lives well for many days in simple media like Ringer-glucose plus antibiotics, and that the helminthicidal action of drugs upon it can readily be tested. When compounds have been selected by this test as having a high degree of activity compared with their probable toxicity, further investigation is of course needed by *in vivo* tests in mice infected with *Hymenolepis* and by detailed studies of oral toxicity.

Results of Testing.—The most active compounds, which kill in our test at a concentration of 1/million, include oil of chenopodium (1 : 10 million), extract of cashew nut, dichlorophen, antimony potassium tartrate and BIQ 20. Of these, oil of chenopodium is well known clinically for its action against ancylostomes and dichlorophen is used successfully for the treatment of tapeworms in dogs. Extracts of cashew nut have been tested clinically for anthelmintic action by Bhaduri, Chakravarti, Bandyopadhyay, Roy, and Arora (1958) with encouraging results ; they have also given further references. Antimony potassium tartrate is too toxic for this kind of therapy, and BIQ 20 is a new compound, synthesized by the workers of Allen & Hanburys and found to possess activity against the filarial worm *Litomosoides carinii* (Hawking and Terry, 1959) ; it will receive further investigation. The

moderately active compounds which kill at a concentration of 1/100,000 include:

male fern extract, hexylresorcinol and mepacrine	which are well-known remedies for human tapeworms.
arecoline	which has been used extensively for tapeworms in dogs, and which is an active depressant of the musculature of cestodes (Batham, 1946; Duguid and Heathcote, 1950a and b).
chloroquine	this is worth further investigation as a drug against tapeworms. It has been reported by Camero (1951) to be effective in removing *Taenia saginata* (7 cases described).
pentaquine and pamaquin	these are also worth further investigation, although their toxicity is greater than that of mepacrine and chloroquine, which would be a disadvantage.
iodochlorhydroxyquin	this would be worth investigating whether it acts on tapeworms as well as on amoebae.
acriflavine	this would presumably be too irritating and too toxic.

Tetrachlorethylene and carbon tetrachloride are difficult to test *in vitro* because of their insolubility and they gave irregular results, the minimum lethal concentrations varying from 1:1,000 to 1:100,000 in different experiments; but when tested *in vivo* tetrachlorethylene given in low dosage seemed moderately active. This is clearly one of the cases in which the *in vitro* test yields equivocal results and further investigation by *in vivo* tests is necessary.

The remaining compounds tested are inactive. In the case of phenothiazine, the inactivity may be due to the insolubility of the compound in our medium. Santonin, although clinically effective against *Ascaris*, is not effective in expelling tapeworms. The other compounds would not be expected to be active upon cestodes. On the whole, the results from our *in vitro* test are in agreement with those obtained by clinical experience.

Grateful acknowledgments are due to Dr. B. Mukerji for encouragement and for facilities for this work; to Dr. O. D. Standen and Mr. J. E. D. Keeling, Wellcome Laboratories of Tropical Medicine, London, N.W.1, for the strain of *Hymenolepis*; to Allen & Hanburys for compound BIQ 20; to Mr. Ram Govind for the extract of *Anacardium*; and to Mr. A. Dutta for technical assistance.

References

Batham, E. J. (1946). *Parasitology*, **37**, 185.
Bhaduri, N. V., Chakravarti, R. N., Bandyopadhyay, A. K., Roy, A., and Arora, U. S. (1958). *Bull. Calcutta Sch. trop. Med.*, **6**, 169.
Camero, C. A. (1951). *Rev. Fac. Med. Bogota*, **20**, 74.
Duguid, A. M. E., and Heathcote, R. St. A. (1950a). *Arch. int. Pharma odyn.*, **82**, 309.
—— —— (1950b). Ibid., **84**, 159.
Hawking, F., and Terry, R. J. (1959). *J. Pharm. Pharmacol.*, **11**, 94.
Sen, A. B., and Hawking, F. (1959). *Proceedings of the Symposium on Amoebiosis and other Intestinal Infections, Lucknow*, November, 1959.
Steward, J. S. (1955). *Parasitology*, **45**, 255.
Watkins, T. I. (1958). *J. Pharm. Pharmacol.*, **10**, 209.
Whitten, L. K. (1956). *Vet. Rev. Annot.*, **2**, 1.