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THE CHEMOTHERAPEUTIC ACTION OF PHENANTHRIDINE COMPOUNDS

PART IV

ACTIVITY *IN VITRO*

BY

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In Parts I, II, and III of this work results have been given of testing a large series of phenanthridine compounds against trypanosome infections in mice. The effect of a drug in an infected animal depends upon the rates of absorption, distribution, metabolism, and excretion, and very little is seen of the way in which the drug acts ; some light should be thrown on this by *in vitro* studies.

Fig. 1 shows the effects of dimidium bromide on infections in mice with four different species of trypanosome. Dimidium bromide differs from other trypanocidal drugs in that the number of trypanosomes in the peripheral blood remains high for a longer time after treatment, and may even increase before it begins to decline to zero. It is also of interest that, although a much larger dose of a phenanthridine compound is necessary to cure an animal infected with *Trypanosoma rhodesiense* than with *T. congolense*, the pattern of the clearance of the trypanosomes from the peripheral blood is very similar. Investigation of the blood levels of these drugs after injection has shown that by the time the maximum trypanocidal action is apparent it is difficult to detect any of the drug in the peripheral blood (Brownlee and Short).

Consideration of these points gave rise to the following questions:

1. Is the action of the phenanthridines a direct trypanocidal one ?

2. Is the short exposure to the initial relatively high concentration of the drug sufficient to damage the trypanosomes so that after one or two generations the power of survival and multiplication is lost ?

3. Is the drug concentrated within the trypanosomes when they are exposed to low concentrations, as has been shown for acriflavine and stilbamidine ?

4. Is the drug activated by contact with the tissues of the animal ?

In studies on *T. cruzi* also, it was of interest to know whether any direct effect by active compounds on the parasite and particularly on the dividing tissue forms could be demonstrated. This organism differs from the other haemoflagellates studied, in that multiplication only takes place inside tissue cells, and not in the peripheral blood. The following experiments were designed to throw light upon these problems.

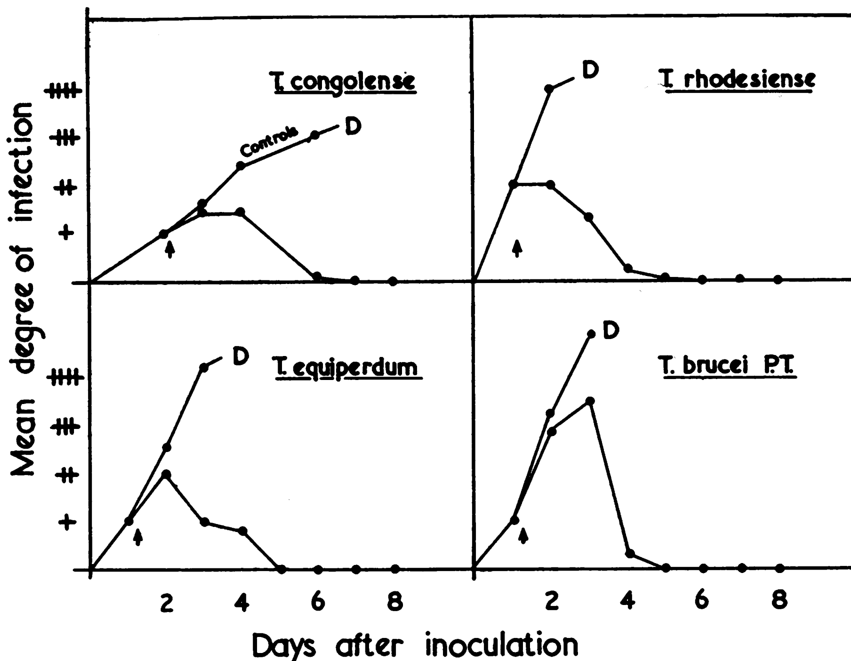


FIG. 1.—Showing slow rate of activity of effective doses of dimidium bromide (6C46) against four strains of trypanosomes in mice. Doses given subcutaneously; *T. congolense*, 0.5 mg./kg.; *T. rhodesiense*, 10 mg./kg.; *T. equiperdum*, 25 mg./kg.; *T. brucei*, 10 mg./kg. Degree of infection assessed from number of trypanosomes seen per microscope field (× 400) in fresh films of peripheral blood: + = 1 in from 1 to 10 fields, ++ = 1 to 5 per field, +++ = 5 to 50 per field, ++++ = more than 50 per field. D = mouse dead.

METHODS

In vitro *experiments with* T. rhodesiense *and* T. cruzi (*blood forms*)

T. rhodesiense.—This species was used for the experiments because of difficulties in getting satisfactory growth of *T. congolense in vitro*. We used a slight modification of the method of Yorke, Adams, and Murgatroyd (1929) for maintaining trypanosomes at 37° C. for 24 hours in undiminished numbers. The medium consisted of equal parts of fresh rabbit serum, sterilized by filtration, and Tyrode solution (NaCl, 0.8 ; KCl, 0.02 ; $CaCl_2$, 0.02 ; $MgCl_2$, 0.02 ; NaH_2PO_4, 0.005 ; $NaHCO_3$, 0.1 ; and glucose 0.4 g. per 100 ml.). Dilutions of drugs were prepared aseptically in the Tyrode solution and distributed in small screw-capped bottles in 0.5-ml. quantities. A suspension containing about 2,000 trypanosomes per μl. in rabbit serum was prepared by centrifuging the citrated blood of an infected mouse. At the start of the test this suspension was kept on the bench at room temperature; 0.5 ml. amounts were added to a series of tubes containing different drug dilutions; a sample was withdrawn from each well-mixed suspension for counting and the tubes placed in an incubator at 37° C. Further samples were then withdrawn at intervals. At least three tubes were used for each concentration of drug. Mean counts were expressed as percentages of the initial count and the results plotted against time.

Considerable variation in the length of life of the controls was observed, but this could not be traced to a definite cause. The same variation was found whether serum

from the same or different rabbits was used in successive experiments. Buffering with carbon dioxide, the *p*H being maintained between 6.2 and 7.8, and the use of heparin in place of citrate as an anticoagulant in the separation of the trypanosomes, did not influence the results.

T. cruzi.—The method used was similar to the above, but the medium consisted of 20 per cent (v/v) of rat serum and 10 per cent (v/v) of chick embryo extract in Tyrode's solution.

The effect of short exposure to drugs upon infectivity

In vitro.—0.1-ml. amounts of suspensions of *T. rhodesiense* from *in vitro* experiments were centrifuged, the trypanosomes washed with drug-free medium, and inoculated into groups of three mice. Daily blood examinations were made for the presence of trypanosomes.

In vivo.—A mouse heavily infected with *T. congolense* was taken and the tail blood inoculated into normal mice. The original mouse was then given drug intravenously and further subinoculations made into fresh mice at intervals.

The effect of relatively short exposure to drug on the survival of trypanosomes in vitro

T. rhodesiense was suspended in various concentrations of drug for varying lengths of time *in vitro*. The trypanosomes were washed and re-suspended in drug-free medium, and the survival times observed. Controls were set up in drug-free medium in the same way.

Activation of the drug

In vitro.—A quantity of sterile rabbit serum was stored at 4° C.; from this small amounts were added to equal volumes of Ringer's solution containing drug and placed in the incubator at 37° C., at various times during a week. On the last day a drop of suspension of trypanosomes was added to each tube and to one prepared from the serum without previous incubation with the drug. The tubes were kept at 37° C. and counts were made at intervals.

In vivo.—Rabbits weighing about 2.5 kg. were injected intravenously with 20 mg./kg. of drug, given very slowly. Blood was taken from a vein in the other ear at intervals, and trypanosomes added to the serum prepared from it. Counts were made, and samples injected at intervals into mice to test for infectivity.

Absorption of drugs by trypanosomes

Trypanosomes from heavily infected rats were suspended in rabbit serum in a concentration of about one million trypanosomes per μl. Quantities of 0.5 ml. of suspension were added to tubes containing 0.5 ml. of various concentrations of drugs in Tyrode's solution. The tubes were incubated at 37° C. for 30 minutes, the trypanosomes then centrifuged down, and the supernatant fluid taken for estimation of drug. The trypanosomes were examined for motility. Estimations of drug were made by the method described in Part III of this work (Goodwin, Goss, and Lock, 1950).

The effects of drugs upon T. cruzi *in tissue culture*

The method of maintaining *T. cruzi* in tissue culture was similar to that described by Hawking (1946), with a modification in the method of infection. Explants from the hearts of embryo rats were placed in a thick suspension of *T. cruzi* in serum-Tyrode mixture. After 10 minutes the explants were removed and stuck on to coverslips in Carrel flasks with drops of fowl plasma. When the plasma had solidified, medium consisting of 20 per cent rat serum and 10 per cent chick embryo extract in Tyrode solution

was added. The *p*H was adjusted to 7.4 by means of a carbon dioxide and air mixture, phenol red being used as an internal indicator. After 10 days at 37° C. considerable numbers of flagellated blood forms were present in and around the explants and many cells contained intracellular forms. Drugs were added in various concentrations to the flasks and allowed to remain there for 48 hours, after which the medium was replaced by drug-free medium. Explants were withdrawn every one or two days subsequently, until the flasks were exhausted. Examinations for moving trypanosomes were made directly, and for intracellular forms after fixing with Schaudinn's solution and staining with Giemsa.

Drugs used in these experiments

The drugs used in these experiments are listed in Table I, which includes the relative *in vivo* activities against *T. rhodesiense* recorded in Part I (Brownlee *et al.*, 1950).

RESULTS AND DISCUSSION

The effects of various dilutions of dimidium bromide (6C46) on *T. rhodesiense in vitro* are shown in Fig. 2. Curves of a similar type were obtained with 129C46, 150C46, and 1C46. Table II shows the dilutions of drugs required to kill 90 per cent of the trypanosomes in 24 and 48 hours, and also the minimum dilutions having no appreciable effect in 72 hours.

TABLE I

LIST OF DRUGS USED AND THEIR RELATIVE *in vivo* ACTIVITIES AGAINST *T. congolense* AND *T. rhodesiense* INFECTIONS IN MICE

Ref. No.	Drug	Relative activity *in vivo*	
		T. congolense	*T. rhodesiense*
150C47	2: 7-diamino-9-*p*-aminophenyl-10-methyl-phenanthridinium chloride	1.5	4.0
129C46*	7-amino-9-*p*-aminophenyl-10-methyl-phenanthridinium bromide	0.3	0.15
6C46†	2: 7-diamino-9-phenyl-10-methyl-phenanthridinium bromide	1.0	1.0
1C46	7-carbethoxyamino-9: 10-dimethyl-phenanthridinium bromide	Practically nil	0.01
3C47	2-amino-9-*p*-carbethoxyaminophenyl-10-methyl-phenanthridinium bromide	0.3	0.5

* Phenidium bromide ("897") † Dimidium bromide ("1553")

TABLE II

DILUTIONS OF DRUGS HAVING COMPARABLE TOXIC EFFECTS ON *T. rhodesiense in vitro*

Drug	Dilution to kill 90% *T. rhodesiense*		Dilution without effect in 72 hrs.
	In 24 hours	In 48 hours	
150C47	1/1,000,000	1/2,000,000	—
129C46	1/750,000	1/1,500,000	1/4,000,000
6C46	1/1,000,000	1/2,000,000	1/4,000,000
1C46	1/250,000	1/500,000	1/1,000,000

TABLE III

THE EFFECT OF 129C46, 6C46, AND 1C46 *in vitro* ON THE INFECTIVITY OF *T. rhodesiense* TO MICE

+++ = 3 mice became infected out of 3 inoculated.
++ = 2 ,, ,, ,, ,, ,, ,,
+ = 1 mouse ,, ,, ,, ,, ,,
− = No mice ,, ,, ,, ,, ,,

The number in parentheses shows the percentage of trypanosomes surviving.

Drug	Dilution	Time of exposure of trypanosomes to drug (hours)											
		0	1	2	3	4	5	6	7	8	9	22	24
129C46	1/100,000	+++(100)	−	−	−	−	−(99)	−	−	−(97)			
	1/300,000	+++(100)	+++	+	++	+	−	−	−	−(90)			
	1/2,000,000	+++(100)		+++		+++		+++(105)				−(5)	−(4)
	1/4,000,000	+++(100)		+++		+++		+++(110)				+++(27)	+++(23)
6C46	1/50,000	+++(100)	−	−	−	−	−	−	−	−(100)			
	1/300,000	+++(100)			+++	+	−	−(100)	−	−(50)			
	1/2,000,000	+++(100)			+++		+++(105)		+++(110)			++(60)	+(55)
	1/4,000,000	+++(100)			+++(97)		+++(105)		+++(100)			+++(30)	+++(28)
1C46	1/100,000	+++(100)	+++	+++		+	++	−	−	−	−(90)		
	1/600,000	+++(100)	+++	+++		+++	+++	+++	+++	+++	+++(90)		
Controls		+++(100)	+++(100)	+++	+++		+++(98)	+++	+++	+++		+++	+++(110)

The drugs exerted a direct lethal effect on the trypanosomes *in vitro* and with those studied the orders of activity *in vitro* and *in vivo* for *T. rhodesiense* were the same. However, Tables I and II show differences in the relative potencies of drugs tested *in vitro* and *in vivo*; thus, 1C46, which shows practically no activity *in vivo,*

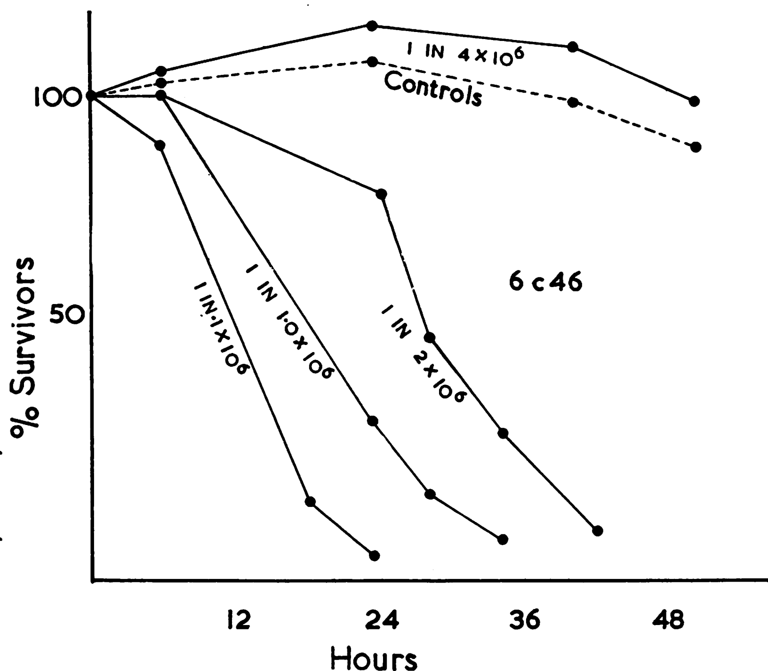


FIG. 2.—The effect of various dilutions of dimidium bromide (6C46) on the survival of *T. rhodesiense in vitro* at 37° C.

has an *in vitro* activity only one quarter that of 150C47, the most active compound of this series. Determination of blood levels of these drugs (Brownlee and Short) indicates that varying rates of absorption and excretion are not sufficient to explain these differences.

Brownlee and Short have also shown that after intravenous doses of these drugs, blood concentrations fall within three or four hours to very low levels, which then persist for at least 24 hours. When trypanosomes were exposed *in vitro* for half or one hour to dilutions of the drugs already shown by the experiments of Table II to be effective in 24 hours, and were then washed free from drug and suspended in drug-free medium, the organisms showed mortality curves similar to those obtained when the drug remained present. Examples of this are shown in Fig. 3. Further evidence of the rapid and persistent effect of 6C46 is shown in Fig. 4. The reduction of infectivity of *T. congolense* taken from an infected animal even half an hour after it had received a dose was well marked, although the trypanosomes remained active and in undiminished numbers in the treated mouse for more than 24 hours.

Table III shows the loss of infectivity of *T. rhodesiense* exposed *in vitro* to various drugs; here again the order of effectiveness is similar to that of the curative action *in vivo*. It seems likely that even if an effective concentration in the blood is maintained for only a short time, this is sufficient to harm the parasites in such

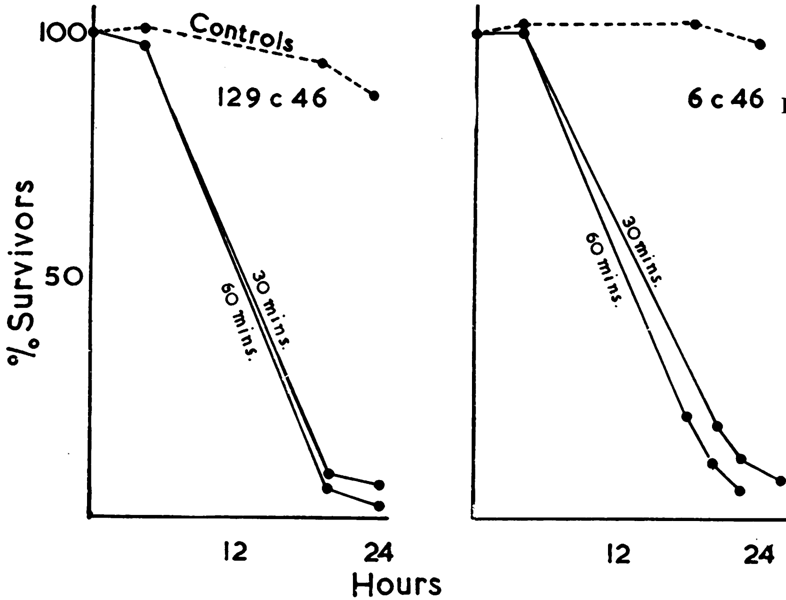


FIG. 3.—Mortality curves for *T. rhodesiense* suspended in drug-free medium after exposure for varying times to 129C46 (1/2,000,000) and 6C46 (1/500,000). Controls received identical treatment but no drug.

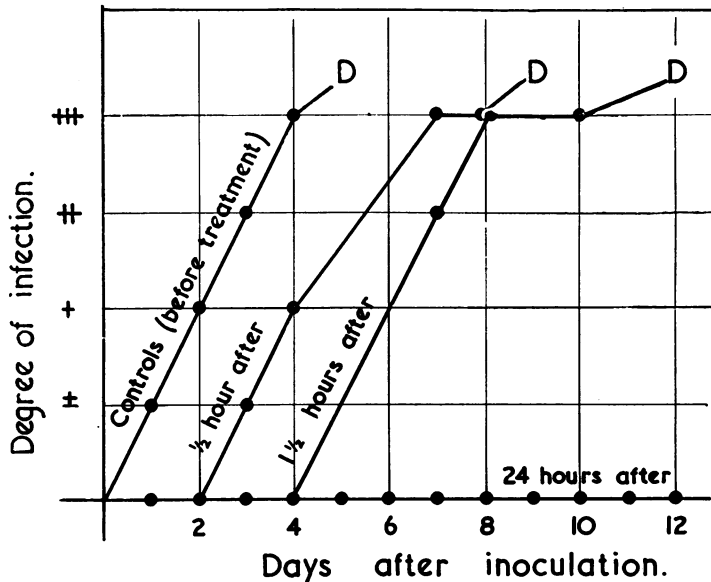


FIG. 4.—The development of *T. congolense* in mice subinoculated from an infected animal which had been treated with 10 μg. 6C46 intravenously. Subinoculations made 0.5, 1.5, and 24 hr. after treatment. Degree of infection as in Fig. 1.

a way that they eventually die. Prolonged exposure to very low concentrations (Fig. 2), even when continued for 72 hours *in vitro*, did not kill the trypanosomes.

The results so far discussed indicate the possibility of rapid absorption and fixation of the drugs by the trypanosomes, but as shown by Table IV, little or no absorption by living organisms could be demonstrated except in a concentration of

10 mg. per 100 ml. In this concentration, however, at the end of 30 minutes many trypanosomes were seen to be moribund. Dead trypanosomes (Table IV) absorbed appreciable amounts of the drugs and it is possible that the absorption shown with

TABLE IV

THE ABSORPTION OF 129C46 AND 6C46 BY *T. rhodesiense in vitro*, ALIVE, AND KILLED BY THREE MINUTES' EXPOSURE TO 60° C.

Drug	Concentration of drug in mg. per 100 ml. in medium			Mg. drug absorbed by 10^8 trypanosomes	
	Initial	Final			
		Living	Dead	Living	Dead
129C46	10.0 5.0 2.5	9.6 5.1 2.5	8.06 4.20 1.66	0.4 0 0	3.88 1.60 0.68
6C46	10.0 5.0 2.5	9.7 5.06 2.46	7.90 4.10 1.66	0.3 0 0.04	4.20 1.80 0.68

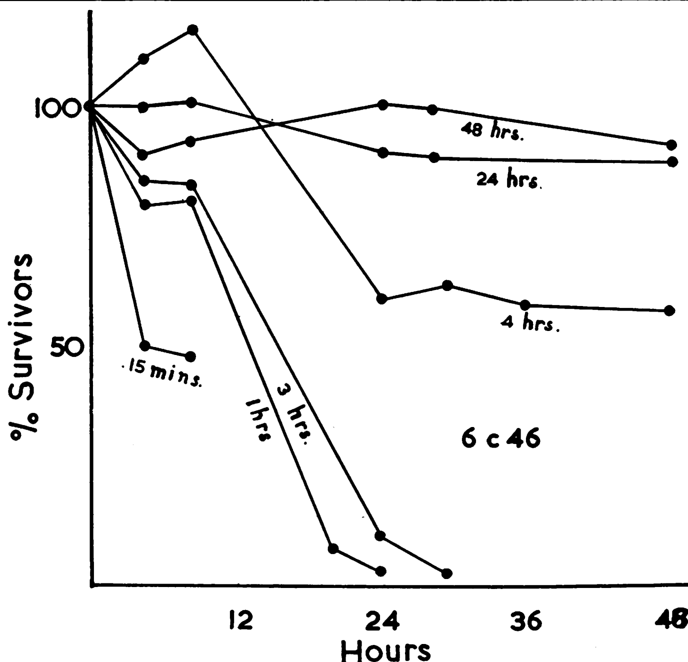
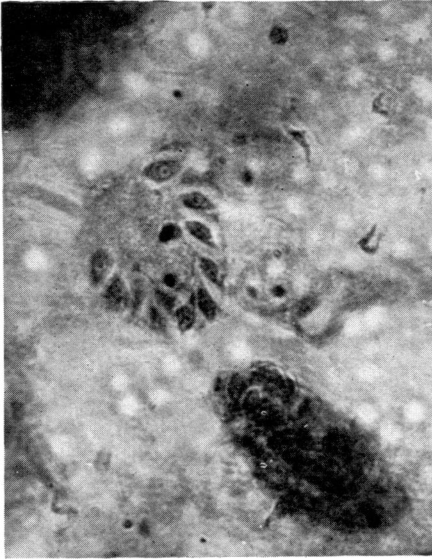


FIG. 5.—Mortality curves for *T. rhodesiense* in rabbit serum taken at intervals from an animal which had received 20 mg./kg. of 6C46 intravenously.

high concentrations resulted from the presence of dead and dying organisms. Under the microscope, living trypanosomes remained unstained but dead ones were immediately coloured by the drugs.

Attempts to demonstrate activation of 6C46 and 129C46 by incubation with serum failed. Similarly (Fig. 5), no *in vitro* antitrypanosomal activity, other than



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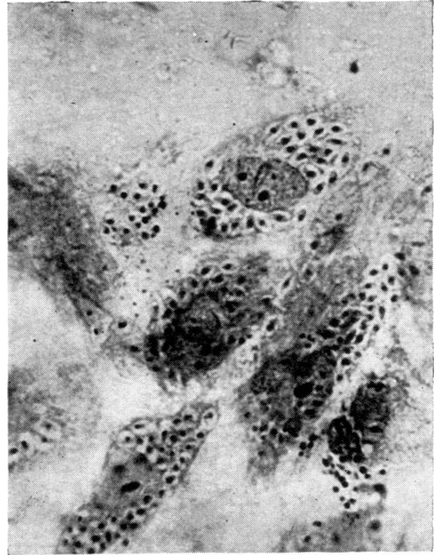

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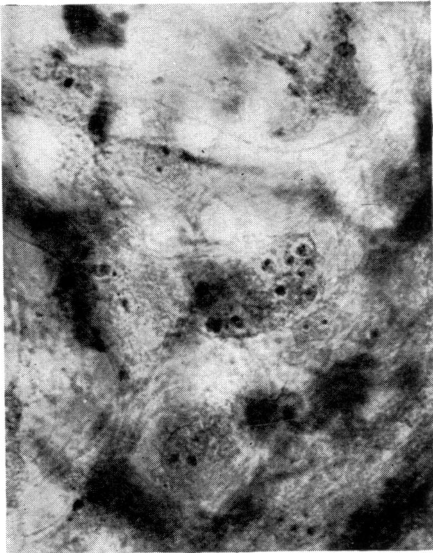

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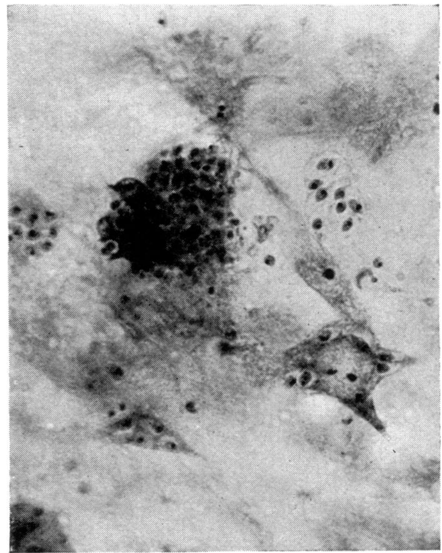

d

PLATE I

that attributable to circulating drug, could be detected in the serum taken from treated rabbits.

Experiments with *T. cruzi* showed that many phenanthridinium compounds possessed activity *in vitro* against the blood forms of *T. cruzi* in dilutions similar to those active against *T. rhodesiense*; results for the *in vitro* activity of 6C46 and 3C47 are shown in Table V. The first of these drugs has been shown to be active

TABLE V

THE EFFECT OF 3C47 AND 6C46 ON THE BLOOD FORMS OF *T. cruzi in vitro*

Drug	Dilution to kill 90% trypanosomes	
	In 24 hours	In 48 hours
3C47	1/150,000	1/70,000
6C46	1/300,000	1/150,000

in vivo against *T. cruzi* (Goodwin, Goss, Lock, and Walls, 1950) when given during the incubation period, but not against an established infection, whereas 3C47 is active under both conditions. It seems probable from this evidence that although 6C46 is toxic to the blood forms of the parasite, it is either inactive against the intracellular forms, or does not penetrate readily enough to produce a concentration lethal to the enclosed parasites. Tissue cultures containing a large number of infected cells (Plate I*a*) exposed to 0.3 and 0.6 mg. per 100 ml. of this drug for 48 hours, subsequently showed some diminution in the rate of increase of infection throughout the host tissues, but development of the intracellular forms continued and flagellates were present in and outside the host cells up to the end of the experiment, five days after replacement of drug-containing by drug-free medium. In both concentrations, however, some effect was seen upon the intracellular forms. Although multiplication and development of the parasites within the cell continued, a proportion degenerated. The nucleus, which in healthy parasites consists of a sphere of chromatin surrounding a clear area containing a central karyosome (Plate I*a*), degenerated to a small irregular mass of deeply staining material; the cytoplasm became shrunken and indistinct (Plate I*b*). However, the normal division and development of the intracellular forms exceeded the rate of destruction and the infection progressed. This occurred even with the cultures exposed to the higher concentration of drug, in which the growth of the host cells was strongly retarded. In this concentration the nuclei of many host cells degenerated and dividing forms were not seen.

Exposure of cultures to 3C47 (0.15 and 0.3 mg. per 100 ml.) produced a rapid cessation of division and development of the intracellular forms. Moving flagellates were absent three days after the removal of the drug. The intracellular forms rapidly became shrunken, there was no evidence of division, and by the fifth day they were difficult to find; those present appeared to be moribund or dead (Plate I*c*). The host cells were apparently unaffected by the drug in these concentrations. Control cultures (Plate I*d*) at the end of the experiment contained large numbers of all forms of the parasite. It is apparent that 3C47 has a direct lethal effect on the intracellular forms of *T. cruzi*.

SUMMARY

1. The effect of some phenanthridine compounds upon *T. rhodesiense* and *T. cruzi in vitro* at 37° C. has been investigated.

2. The drugs were shown to be effective in high dilution *in vitro* and the order of activities *in vitro* and *in vivo* are similar. However, the effective concentration *in vivo* of a really potent compound appears to be much lower than the effective concentration *in vitro*, and considerable activity *in vitro* is shown by a compound which has no significant activity *in vivo*.

3. A short exposure to an effective drug *in vitro* or *in vivo* profoundly affected the power of survival of *T. rhodesiense* and *T. congolense*.

4. No appreciable absorption of the drugs by living trypanosomes could be detected by the methods at our disposal.

5. No activation of the drugs *in vivo* or *in vitro* could be found.

6. A direct toxic effect of a drug which is active *in vivo* has been shown on the intracellular forms of *T. cruzi* in tissue culture.

My best thanks are due to Miss R. Shipman, Messrs. P. Bradley and M. W. C. Cheeseman for assistance, to Mr. J. W. Michaeli for taking the photomicrographs, and to Mr. L. G. Goodwin for much advice and encouragement.

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