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A STUDY OF RESISTANCE TO ANTRYCIDE IN A STRAIN OF *TRYPANOSOMA EQUIPERDUM*

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In a previous paper on the mode of action of antrycide (Ormerod, 1951a) an antrycide resistant strain of *Trypanosoma equiperdum* was used. This paper describes: (1) Methods used for preparing resistant strains and the way in which they acquired resistance. (2) Experiments designed to test the effect of drugs on infections produced by normal and resistant strains, and the mode of absorption of drugs by the two strains. (3) A change that occurred in the normal strain which was accidentally treated with a dye having mild trypanocidal activity.

The findings are discussed in relation to the hypothesis (Ormerod, 1951b) that antrycide and several other "trypanocidal" substances inhibit the growth of trypanosomes by splitting the cytoplasmic nucleoprotein into its constituent protein and ribonucleic acid, thereby preventing the interaction of these substances necessary for growth and reproduction.

MATERIALS

A normal strain of *T. equiperdum* used at the National Institute for drug standardization was employed during the course of this work; initially the cytoplasm was clear when stained with eosin-methylene blue compound stains and there was not more than one inclusion per ten trypanosomes when viewed in the living active state by phase contrast microscopy. Antrycide was provided by Messrs. Imperial Chemical (Pharmaceuticals) Ltd. as the methylsulphate. The chloride was prepared by precipitation from a solution of the methylsulphate by adding sodium chloride. "Collosol Cuprum," Crookes brand of 0.05 per cent colloidal copper, was used in normal and splenectomized mice according to the method of Hawking (1939) and von Jancsó (1934) for "reticulo-endothelial blockade."

RESULTS

The preparation of resistant strains

Resistant strain I.—At the first attempt to form a resistant strain, one of a group of twelve mice with well established infections, previously given 0.3 ml. intravenous collosol cuprum, failed to respond to 0.01 mg./20 g. of antrycide given intraperitoneally. After 7 days 0.1 mg./20 g. of antrycide would not control this infection, which was transmitted 8 times successively to clean mice each dosed with 0.1 mg./20 g. of antrycide. The intraperitoneal dose that could then be given without controlling the infection was 0.25 mg./20 g. (the LD50 in mice by this route is 0.34 mg./20 g.). This dose, however, given on two consecutive days would cure the infection.

Rough tests using two groups of 4 mice showed no gross differences between normal and resistant strains in response to dimidium bromide and suramin.

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The strain was stored for a month in guinea-pigs, and during this time lost completely its resistance to antrycide.

Resistant strain II.—The same technique was employed to produce another resistant strain, but when after 6 transmissions in 30 days no increase in resistance had occurred, it was modified in the following way. Splenectomized mice with a well established infection were given an intravenous injection of 0.2 ml. colloidal copper followed by a subcutaneous injection of antrycide chloride. The insoluble antrycide chloride was given as a suspension of crystals, 1 mg./ml. in 10 per cent saline, so as to ensure the slowest possible absorption of the drug into the blood stream. A group of 4–6 mice was maintained in this way, infected, and treated daily ; and two fresh mice were added to the group every one or two days to replace those that had died or had been cured. At each transmission, all the mice were infected one from another. The essentials of this technique were: the maintenance of a high trypanosome count, frequent transmissions, and prolonged action of small amounts of the drug while there was as yet little resistance. When the increase in resistance seemed to warrant it, this technique was abandoned, and infected mice were dosed with antrycide methylsulphate without previous splenectomy or injection of colloidal copper.

The progress of resistance of the strain under treatment with antrycide is shown in Table I. The minimum number of possible transmissions that the resistant strain

TABLE I

PROGRESS OF RESISTANCE

Table showing the increase in resistance of the strain with the treatment outlined in the text. Fifty per cent lethal dose of antrycide methylsulphate intraperitoneally $\simeq$0.34 mg./20 g. mouse. Fifty per cent curative dose of antrycide methylsulphate intraperitoneally on the normal parent strain $\simeq$ 0.013 mg./20 g. mouse.

No. of mice added to antrycide treated group	Days from beginning of experiment	Maximum intraperitoneal dose that failed to clear the blood mg./20 g.
0	0	0.01
17	46	0.05
31	69	0.1
45	89	0.1
45	96	0.2
45	99	0.1
55	106	0.5
55	110	0.2
60	126	0.8 (in four doses)
	191	} No influence on infection
	548	
	Treatment with antrycide stopped	
	594	0.05 (cleared the blood)

could have undergone is equivalent to the number of mice added to the treated group ; as each mouse was infected approximately ten times, the maximum possible number of transmissions is of the order of ten times this figure. As in resistant strain I, each increase in resistance occurred suddenly at one point in the series of transmissions, and although the technique of cross-infection between all the animals made this point difficult to define, it was clear that there was no gradual transition from susceptibility to resistance. Another point of interest in the progress of this strain from susceptibility to resistance was that loss of resistance could occur during

treatment as well as gain ; for instance, on the 106th day of the experiment trypanosomes from one mouse in the group required more than the LD50 of antrycide methylsulphate, whereas trypanosomes from another mouse in the group with a previously identical infection gave a 50 per cent cure (determined graphically from a 6-point assay) with 0.062 mg./20 g. mouse, only 2.5 times the original 50 per cent curative dose.

As is shown in Table I, the necessary curative dose 106 days after the beginning of the experiment exceeded the LD50 of the drug for mice ; from then on resistance continued to increase so that the infection was no longer influenced by repeated injections of the maximum tolerated dose of antrycide ; at this point the experiments described below were performed. Throughout their progress animals bearing the resistant strain were dosed with antrycide at each passage ; but after the experiments finished (548 days) the strain was no longer dosed and lost its resistance in a further 46 days, so that the blood of a mouse infected at the rate of one trypanosome per one-twelfth-inch oil-immersion microscopic field could be cleared by 0.05 mg./20 g. of antrycide methylsulphate.

Experiments with the fully resistant strain

Cross resistance tests.—As there was no demonstrable difference in infectivity between the normal and antrycide resistant strains, it was possible to determine the degree of resistance of the antrycide resistant strain as compared with the parent strain for any particular drug by comparing the log dose-response lines that it produced using the two strains in simultaneous experiments under identical conditions. It was assumed for this purpose that when the blood trypanosome counts of the two groups of mice were similar, any difference in response of the strains was due to their difference in resistance to the drug in question.

Mice of 18–21 g. were infected the previous day with a number of trypanosomes, approximately 200 million, adjusted to give an infection at the rate of one trypanosome per one-twelfth-inch oil-immersion field on the day of the experiment ; animals with a significantly different degree of infection were rejected from the experiment. The blood films were examined in each experiment at the point found to give the greatest difference in response between high and low doses of the drug: this was found to be at 48 hours for dimidium bromide and at 24 hours for the other drugs studied. The results with suramin were found to be best at 24 hours despite the fact that the blood of many mice still infected at 24 hours would have been cleared in 48 hours. Wet smears of approximately the same density were made from tail blood. Failure to see one trypanosome in 20 fields using a one-twelfth-inch oil-immersion objective was taken as a positive response. This relatively crude method was found to be as satisfactory as any other available for determining whether the blood of an animal was clear or infected. No account was taken of subsequent relapse or clearing of the blood, since the sole object of the experiment was to obtain satisfactory graded log dose-response lines from which the difference in resistance of the two strains could be estimated.

The results of these tests are shown in Table II. Dimidium and stilbamidine were, like antrycide, unable to clear trypanosomes from the blood of mice within the limits set by acute toxicity of these drugs, and the antrycide resistant strain may to this extent be said to be totally resistant to them. No resistance could be demonstrated to suramin: the figures suggest an increased sensitivity of the antrycide resistant strain to this drug, but do not admit of analysis for significance. The

TABLE II

Table showing the degree of cross-resistance of antrycide resistant strain II to the drugs shown in the first column. Resistance in the last column is the reciprocal of "Potency" in the usual type of drug assay. One experiment each with trypan blue and *p*-hydroxyphenylarsenoxide could be assessed statistically. With acriflavine the three experiments were combined before assessment.

Drug	Dose mg./20 g.	Normal Strain		Resistant strain		No. of deaths from drug toxicity	Resistance as compared with normal strain = 1 (fiducial limits)
		No. cleared	Total	No. cleared	Total		
Suramin:							
Exp. (i)	0.020	0	4	0	4		
	0.028	0	4	3	4		
Exp. (ii)	0.020			5	5		
	0.028	4	5	4	4		
	0.039	5	5				
Exp. (iii)	0.020	0	5	0	5		
	0.025	0	6	1	6		
	0.030	1	6	4	6		
	0.035	1	5	2	5		
Trypan blue:							
Exp. (i)	0.7	0	10	1	10		
	0.8	1	10	7	10		0.877
	0.9	4	10	6	10		(0.76–1.08)
	1.0	7	10	7	10		
Exp. (ii)	0.5	0	6	0	6		
	0.6	0	6	3	6		
	0.7	0	6	3	6		
	0.8	0	6	3	6		
	0.9	2	6	4	4		
p-Hydroxyphenyl-arsenoxide:							
Exp. (i)	0.015	0	5	0	5		
	0.017	2	5	2	5		1.09
	0.020	4	5	2	5		(0.85–1.31)
	0.0225	5	5	4	5		
Exp. (ii)	0.01	0	6	0	6		
	0.02	4	6	5	6		
	0.03	6	6	6	6		
Acriflavine:							
Exp. (i)	0.15	0	10				
	0.19	2	10				
	0.24	2	9				
	0.25			0	10		
	0.31			3	9		
	0.39			6	10		
Exp. (ii)	0.19	3	10				
	0.24	9	10				
	0.25			3	10		1.31
	0.30	10	10				(1.19–1.44)
	0.31			8	10		
	0.39			10	10		
Exp. (iii)	0.19	0	9				
	0.24	2	8				
	0.25			0	9		
	0.30	6	8				
	0.31			4	9		
	0.39			10	10		

TABLE II—*continued*

Drug	Dose mg./20 g.	Normal strain		Resistant strain		No. of deaths from drug toxicity	Resistance as compared with normal strain=1 (fiducial limits)
		No. cleared	Total	No. cleared	Total		
Dimidium	0.2	0	8			0	
	0.4	3	5			0	
	0.5	1	5	0	5	0	
	0.7	2	5			0	
	0.8	3	7	0	5	0	>2.3*
	1.0	4	5			0	
	1.25			0	5	1	
	1.28			0	4	1	
	1.6			0	5	2	
	2.05			0	10	7	
Stilbamidine ..	0.48	4	10			0	
	0.57	8	10			0	
	0.69	7	10			0	>3.2*
	1.60			0	10	0	
	2.40			0	10	5	
	3.60			0	10	10	
Antrycide	0.004	1	10				
	0.008	4	10				
	0.016	5	10				
	0.032	9	10				
	0.27				18	3	>26.0*
	0.32				18	10	

* LD50/ED50 for normal strain.

figures for trypan blue in the same way suggest an increased sensitivity of the "resistant" strain, but this is not significant. There is no significant difference in the responses of the two strains to *p*-hydroxyphenylarsenoxide. An experiment with tryparsamide was not completed because of the change in the normal strain (described below). A small but statistically significant difference in the response of the two strains to acriflavine was shown, and represented a 1.3-fold increase in resistance.

The absorption of drugs visualized by fluorescence microscopy.—A study was made of the fluorescence of normal and resistant strains after absorption of antrycide. An Osira lamp and a Wrattan 18A filter with transmission maximum in the 3650A region were used. The typical appearance in the normal strain was the same as previously described (Ormerod, 1951a). (The highly fluorescent spot described previously was now identified as the kinetoplast and not the basal vacuole as I had then supposed.) One hour after a mouse had been treated with a large dose of antrycide, 0.2 mg./20 g., the kinetoplast in the trypanosomes of both strains became fluorescent with white light, and the cell body uniformly fluorescent with blue light. After about four hours blue granules appeared in the cytoplasm, most numerous in the resistant trypanosomes. After 24 hours the kinetoplast was still fluorescent but not the cell body of the antrycide resistant trypanosomes, in which only the kinetoplast could be seen; but in the normal trypanosomes the kinetoplast and numerous granules were fluorescent with a white light.

When 2-hydroxystilbamidine was injected into infected mice the effect on the two strains was most striking. At three hours after injection of 0.5 mg./20 g. of 2-hydroxystilbamidine (the time at which there was the greatest differentiation between the two strains) trypanosomes of the normal strain showed a bright yellow kinetoplast and one or more yellow granules of the same size and intensity in the cytoplasm. Trypanosomes of the resistant strain showed the same bright yellow kinetoplast, but the granules appeared smaller and with a blue fluorescence.

Plates I and II show trypanosomes of the normal and the resistant strains treated with 2-hydroxystilbamidine. In both strains the kinetoplast fluoresces orange ; in the resistant strain the small granules (which are already present in the untreated organism) have absorbed the drug and produce a blue fluorescence. On the other hand, the large granules which appear in the normal strain are produced by the drug which gives a brilliant orange fluorescence fading little on exposure to ultra-violet light. Similar changes occurred in trypanosomes treated with stilbamidine giving the same distinction between normal and resistant strains.

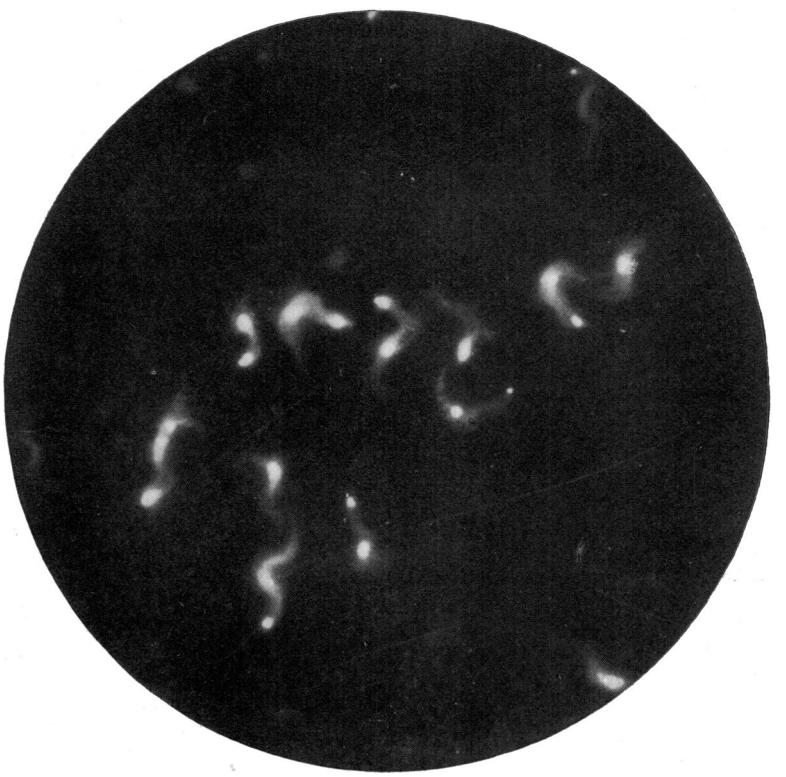

PLATE I.—Normal *T. equiperdum* in a dry unstained blood smear taken 3 hours after 0.5 mg./20 g. 2-hydroxystilbamidine. Both kinetoplast and inclusions produced a brilliant orange-yellow fluorescence when irradiated with ultra-violet light in the 3650A region.

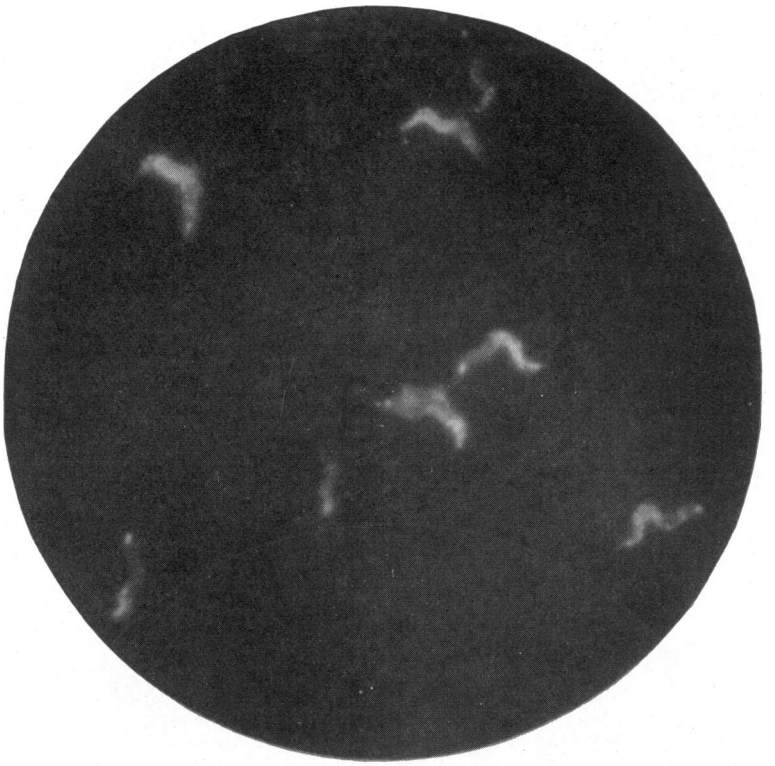

PLATE II.—Antrycide resistant *T. equiperdum* in a dry unstained blood smear taken 3 hours after 0.5 mg./20 g. of 2-hydroxystilbamidine. The kinetoplast produced an orange-yellow and the inclusions a blue fluorescence when irradiated with ultra-violet light in the 3650A region.

The production of granules in a strain that previously produced trypanosomes with agranular cytoplasm

When the antrycide resistant strain had been fully formed, it was noticed that the cytoplasm contained granules whereas that of the normal parent strain was relatively clear when examined by phase-contrast microscopy. These granules could not be stained with eosin-methylene blue compound stains but were otherwise similar to the inclusions produced by drugs. However, an accident occurred in the laboratory which will be described in detail, as it provides an example of the formation of granules while the strain was under microscopic control. On March 19, 1951, the normal strain was examined by phase contrast and inclusions were seen at a rate not exceeding one per ten trypanosomes; on April 3, when the strain was re-examined the cytoplasm of each trypanosome was found crammed with granules of varying sizes. These granules were highly refractile and appeared as purplish spots under ordinary light microscopy, and showed a yellow fluorescence under ultra-violet. They did not stain with eosin-methylene blue compound stains. It was then noticed that the mice were excreting pink urine, and on inquiry it was discovered that since weaning they had been fed on a diet containing the dye, Rhodamine B

(Edicol Rose B) Colour Index No. 749, in a concentration of 1:18,000. A large dose of this dye, 0.5 mg./20 g., was found to clear the blood of trypanosomes, but relapse occurred within two days. It is uncertain for how long the rats and mice in the Institute were receiving this diet, for although this diet was removed as soon as the effect was noted it is likely that traces remained in the bodies of animals for at least a month.

In two months' time when all animals were free from dye, never having received the diet that contained it, the granules still persisted in the trypanosomes; they were, however, no longer coloured or fluorescent, but as numerous and as refractile as ever. A dose of 1.0 mg./20 g. of Rhodamine B (the maximum tolerated dose for mice) would no longer clear the blood, and in several experiments a slight resistance to antrycide could be detected, but its degree could not be evaluated because it seems to have been unstable, and because there was now no *normal* parent strain with which to compare it. Thirteen months later these granules were still present although smaller and in diminished numbers.

DISCUSSION

The use of colloidal copper

The rationale for using colloidal copper in preparing drug resistant strains is based on the theories of von Jancsó and von Jancsó (1935), who found that a dose of colloidal copper antagonized the therapeutic action of suramin on trypanosomes and caused the appearance after suramin of abnormal multinuclear forms. They believed that the action of suramin was to coat the trypanosome in the manner of an "opsonin" so that it could be seized by reticulo-endothelial cells: when the reticulo-endothelial system was poisoned by a dose of colloidal copper the abnormal forms would then be able to survive, and among the trypanosomes that would be spared in this way would be the partially resistant forms. Hawking (1939) has used this technique successfully in preparing a suramin resistant strain, so it seemed reasonable to apply it to the formation of an antrycide resistant strain, all the more since preliminary experiment had shown that "collosol cuprum" had a marked inhibitory effect on the therapeutic action of antrycide.

But soon it became clear that some different mechanism was in operation; the reasons for this were as follows:

(1) Treatment with "collosol cuprum" delayed the appearance of basophilic inclusion bodies which usually occur in trypanosomes within twenty-four hours after an effective dose of antrycide. The inclusion bodies did not occur at all when the therapeutic effect of antrycide was completely inhibited.

(2) Previous intraperitoneal administration of "collosol cuprum" diminished slightly the toxicity of intraperitoneal antrycide methylsulphate.

(3) Antrycide forms a precipitate *in vitro* with "collosol cuprum."

(4) Multinuclear forms similar to those referred to by the von Jancsós were produced (Ormerod, 1951a) in *T. rhodesiense* (without treatment with colloidal copper) by a fifth of the dose of antrycide needed to clear the blood of trypanosomes.

These experiments were not pressed beyond the point where it became likely that a process other than blockade of the reticulo-endothelial system was influencing the increase in antrycide resistance, since although they throw doubt on the conclusions of the von Jancsós, it seemed fruitless to repeat their actual experiments, as they did not indicate the exact nature of the colloidal copper that they used or its method of preparation. In "collosol cuprum" the colloidal copper is stabilized with peptone and cresol; other

stabilizing agents can be used such as egg albumin, gum arabic, or agar, and as some of these might combine with suramin and others not, it is impossible to say whether the therapeutic inhibition that the von Jancsós observed was actually produced by reticulo-endothelial blockade or by fixation of the drug in the way that " collosol cuprum " seems to be able to fix antrycide *in vivo*.

The benefits of using colloidal copper in the production of an antrycide resistant strain—if indeed there are benefits—probably consist in keeping the organisms subjected to a low concentration of the drug for long periods.

The mechanism of antrycide resistance

These experiments, as far as they have been pursued, have shown a distinction in the reactions of the antrycide resistant strain between acidic and basic drugs. No resistance to suramin, trypan blue, or *p*-hydroxyphenylarsenoxide was demonstrable, but to the basic drugs dimidium and stilbamidine there was well-marked resistance, and there was slight resistance to acriflavine.

Most workers who have studied the relative absorption of drugs by normal and resistant strains of trypanosomes agree that the resistant strains show a diminished absorption of the drug ; much of this work is epitomized by that of Hawking (1934, 1937, and 1944), who, using biological and chemical methods of estimation, was able to show that the arsenicals, acriflavine, and stilbamidine were absorbed in considerable quantities by normal trypanosomes, but in smaller amounts by resistant trypanosomes. Suramin was absorbed by the normal strain in such small amounts that he was unable to detect differences in absorption between normal and resistant strains (Hawking, 1939). In this investigation I have not been able to detect such differences in the absorption of antrycide by the two strains beyond the different appearance that the two strains show on examination by fluorescence microscopy, and these cannot be interpreted quantitatively because of the colour and intensity differences. Qualitatively, however, it is clear that antrycide, stilbamidine, and 2-hydroxystilbamidine are able to penetrate the trypanosome cell of the resistant as well as the normal strain, but the normal cell seems to retain the drug in its substance, while it disappears from the resistant strain and does not affect its growth and metabolism. A strong suggestion as to the meaning of the colour differences between the two strains comes from the work of Oster (1951), who showed that a small shift occurred in the wavelength of acriflavine on forming a complex salt with nucleic acid. More spectacular use of the same phenomenon was made by Snapper and others (1951), who showed an even more striking shift in the wavelength of 2-hydroxystilbamidine on combination with ribonucleic acid: the hydrochloride of 2-hydroxystilbamidine produces a faint blue fluorescence, but in combination with nucleic acid it changes to a brilliant orange yellow. Snapper and his co-workers also showed that the brilliant orange yellow fluorescence was given from cell structures known to be rich in ribonucleic acid when the cell had absorbed the drug. While this orange-yellow is certainly not specific for nucleic acids—it is produced by combination of 2-hydroxystilbamidine with other polybasic acids such as heparin and to some extent with ethionic acid—the observations of bright orange-yellow bodies in the normal strain of trypanosomes indicate that the drug is fixed by acidic structures in the cell, and evidence has already been put forward (Ormerod, 1951b) to suggest that such structures are composed largely of protein and ribonucleic acid. The blue fluorescence of the bodies in the cytoplasm of the resistant strain shows that in some way

the drug is prevented from combining with these structures. In this connection it is interesting that Schueler (1947) has suggested a shift in the isoelectric point of cytoplasmic protein of resistant trypanosomes, since he observed differences in staining capacity of drug-resistant trypanosomes at increased hydrogen ion concentrations.

In spite of this clear-cut mechanism it should not be assumed that this is the only way that a trypanosome can acquire drug resistance. The sudden increases in resistance that were observed may represent the acquisition of other mechanisms of drug resistance, and the fact that the resistance of the strain for antrycide is greater than for other drugs may suggest other mechanisms more specific for antrycide.

It is tempting to suppose that the inclusion bodies found by phase contrast examination of the antrycide resistant strain correspond to those produced by rhodamine and are an essential part of the mechanism of drug resistance ; it must, however, be emphasized that these changes were found by accident, and in the one instance the way in which the inclusions developed was not observed, and in the other the conditions under which they developed were not under control.

Conclusion

As a final conclusion it can be stated that nothing in this work has been shown to be inconsistent with the working hypothesis (Ormerod, 1951b) that antrycide and several other drugs inhibit the growth of trypanosomes by splitting the cytoplasmic ribonucleoprotein into its constituent protein and ribonucleic acid. To this group of drugs it is now possible to add stilbamidine and 2-hydroxystilbamidine. True, they behave differently in other ways, as Hawking (1944) has shown that stilbamidine can be trypanocidal *in vitro,* and the inclusion bodies which it produces in less than half an hour's treatment are not basophilic and can only be demonstrated by the fluorescence in the dry smear or by phase contrast in the living organism. A further addition to the hypothesis concerns a mechanism of resistance that has been demonstrated in this work: whereas the nucleoprotein in the cytoplasm of the normal trypanosome is reactive and can be split by drugs, the nucleoprotein of the antrycide resistant organism is for some reason unreactive to antrycide and other basic drugs so that they are able to enter and leave the cytoplasm without damaging the cell or inhibiting its growth.

Summary

1. Two antrycide resistant strains of *T. equiperdum* were prepared using "collosol cuprum." Evidence is given in brief to suggest that the action of "collosol cuprum" is to fix the drug so that the organism is subjected only to small amounts.

2. The resistant strains were unstable and had to be maintained with constant doses of antrycide.

3. Resistant strain II showed cross resistance that was complete—within the limits set by the toxicity of the drug for mice—to dimidium and stilbamidine ; partial resistance to acriflavine was observed, but no resistance to suramin, trypan blue, and *p*-hydroxyphenylarsenoxide.

4. Inclusion bodies were observed in the cytoplasm of the normal strain of *T. equiperdum* that had previously been free from granules but had been treated

accidentally with Rhodamine B. These inclusion bodies could not be stained with eosin-methylene blue compound stains but were demonstrated by phase-contrast microscopy. At first they contained the dye but persisted for many months after its withdrawal in the descendants of the affected trypanosomes.

5. Inclusion bodies with similar properties were observed in the fully developed antrycide resistant strain.

6. A striking differentiation between the normal and resistant strains is produced by treatment with 2-hydroxystilbamidine. In trypanosomes of the normal strain inclusion bodies appear with bright orange fluorescence (these are produced by the drug) and in the resistant strain many blue granules (present in the organism before exhibition of the drug) are shown. Similar but less striking changes occur with antrycide and stilbamidine.

7. A mechanism of drug resistance has been demonstrated in which the cytoplasmic nucleoprotein, which is normally split by basic tryanocidal drugs into protein and a combination of the drug with ribonucleic acid, becomes unreactive so that these drugs can no longer combine with it. They therefore leave the cell when the blood concentration falls, since they are not bound to the cell structure. This is not likely to be the only mechanism whereby a trypanosome can acquire drug resistance, but in this instance it is probably the most important.

I should like to express my thanks to Dr. F. Hawking, who proposed that I should investigate the mode of action of antrycide, for his interest and stimulating criticism; to Mr. J. Smiles and his staff for their interest and co-operation, to which I owe the illustrations and much of the substance of the work; to Dr. W. L. M. Perry, for advice and for the statistical analysis; and to my assistants, Mr. D. Cragoe, Mr. F. Jabir, and Mr. C. B. Hill.

REFERENCES

Hawking, F. (1934). *Ann. trop. Med. Parasit.*, **28**, 67.
Hawking, F. (1937). *J. Pharmacol.*, **59**, 123.
Hawking, F. (1939). *Ann. trop. Med. Parasit.*, **33**, 13.
Hawking, F. (1944). *J. Pharmacol.*, **82**, 31.
Ormerod, W. E. (1951a). *Brit. J. Pharmacol.*, **6**, 325.
Ormerod, W. E. (1951b). *Brit. J. Pharmacol.*, **6**, 334.
Oster, G. (1951). *Trans. Faraday Soc.*, **47**, 660.
Schueler, F. W. (1947). *J. infect. Dis.*, **81**, 139.
Snapper, I., Schneid, B., Lieben, F., Gerber, I., and Greenspan, E. (1951). *J. Lab. clin. Med.*, **37** 562.
von Jancsó, N., and von Jancsó, H. (1934). *Zbl. Bakt. (I. Abt. Orig.)*, **132**, 257.
von Jancsó, N., and von Jancsó, H. (1935). *Ann. trop. Med. Parasit.*, **29**, 95.