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THE MODE OF ACTION OF HETRAZAN ON FILARIAL WORMS

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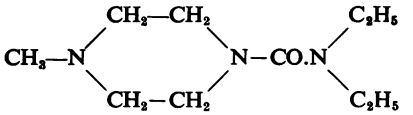
This paper describes an investigation of the antifilarial action of hetrazan in experimental animals, with special reference to the mechanism underlying its action. A preliminary communication on the work was given by Hawking, Sewell, and Thurston (1948).

Hetrazan is—

1-diethylcarbamyl-4-methylpiperazine



The antifilarial action of hetrazan was discovered and described by Hewitt, Kushner, Stewart, White, Wallace, and SubbaRow (1947) working with cotton rats infected with *Litomosoides carinii*, and its action on human filariasis due to *W. bancrofti* was shown by Santiago-Stevenson, Oliver-Gonzalez, and Hewitt (1947). The present experiments were carried out on cotton rats bred at this Institute and infected with *Litomosoides* by the methods described by Hawking and Sewell (1948). The hetrazan used was the dihydrogen citrate, kindly supplied by American Cyanamid Co., and all doses refer to this salt. Microfilaria counts were made by spreading 5 to 20 cu.mm. of blood on a slide to form a thick film, dehaemoglobinizing in water, fixing in alcohol, and staining with hot haemalum or with Giemsa's stain ; the microfilariae were then counted under the microscope.

Preliminary experiments in vivo

Infected cotton rats were given hetrazan by intraperitoneal injection according to the schedules shown in Table I and daily counts were made of the microfilariae in the blood taken from the tail. The rats were treated about seventy days after infection ; during the next month untreated rats usually show a gradual increase in the microfilaria count. According to Harned *et al.* (1948) the LD50 of hetrazan hydrochloride when given intraperitoneally to rats is 465 mg./kg. In our experiments single intraperitoneal doses of 500 mg. of the citrate per kg. or hourly doses 100 mg./kg. usually caused prostration for $\frac{1}{2}$–1 hour after each injection ; repetition of the 500 mg./kg. dose after one day sometimes caused death, but 250 mg./kg. twice daily was well tolerated. The effect on the number of microfilariae in the blood varied considerably in different animals so that precise quantitative results

TABLE I

EFFECT OF HETRAZAN ON ADULT WORMS AND MICROFILARIAE *in vivo*

Each horizontal line refers to one rat. The rats were treated by intraperitoneal injection 64–75 days after the last exposure to infective mites. Among 160 male worms found in the rats of the third and fourth dose-schedules only one was dead.

Schedule	Microfilariae per cu.mm. of tail blood												Post mortem findings	
	Before first dose	Days after first dose											Days after last dose	Female worms No. dead/ total No.
		1	2	3	4	6	7	8	9	10	11–14	20–23	35–40	
Single dose														
500 mg./kg. ..	15	2	1	9	3	7	3	2	4	4	0			14	0/2
200 ,, ..	21	5	3	3	11	15	4	1	4	15	9	21	87	37	4/8
100 ,, ..	37	0	7	10	29	6	24	23						13	0/many
100 ,, ..	10	5	3	5	6	14	7	6						13	Few/many
50 ,, ..	73	12	13	7	9	14	11	17						45	?0/2[1]
50 ,, ..	21	25	4	12	7	6	11	13						51	2/many
20 ,, ..	7	2	0	4	2	4	3	1						44	?All/many[1]
20 ,, ..	18	21	13	13	24	18	21	32						51	0/many
10 ,, ..	16	11	5	14	3	16	18	84						8	2/many
10 ,, ..	15	5	1	4	12	10	9	8						51	0/few
6 *daily doses*															
100 mg./kg. ..	13	13	0	0	1	1	2	1	3	7	4	20		15	1/2
50 ,, ..	63	3	4	1	0	3	2	1	8	8	8	27		Became negative six months later	
20 ,, ..	5	4	5	1	4	1	1	0	2	3	4	16	78	32	0/many
10 ,, ..	53	70	2	2	11	19	1	11						8	0/many
10 ,, ..	31	9	2	7	5	1	7	15						8	2/4
5 ,, ..	2	6	4	3	5	8	16	29						5	0/many
5 ,, ..	143	159	13	13	39	18	20	13						46	0/many
2 ,, ..	31	32	0	8	4	4	14	8						34	0/1
1 ,, ..	24	14	3	2	2	2	0	0						29	0/0
12 *twice daily doses*															
200 mg./kg. ..	8	0	0	2										2	0/3
200 ,, ..	32	0	0		0	4					16			9	1/7
50 ,, ..	16	0	0		0									2	0/14
50 ,, ..	30	0	0		2	6					38			9	0/6
20 ,, ..	4	5	0	8	1	1	3	1	3	4	3	30		15	0/few
10 ,, ..	20	2	2		2						24			9	0/74
10 ,, ..	54	12	10		16						76			9	1/33
300 mg. per kg. Interval 2 hours ; then 8 doses of 100 mg. per kg. at intervals of 1 hour	676	40			36	184								5	?All/many[1]
	22[2]	0							25					10	0/24
	0[2]	0							60					10	0/10
	0[2]	0										42		30	3/9
	2[2]	0										40		30	3/9

[1] Rat died. [2] Rats treated at 46th day after exposure to mites, when microfilariae had hardly reached the blood.

were hardly obtainable. Single doses greater than 50 mg. per kg. usually caused a marked diminution of the microfilaria count which often passed off after 4–8 days. Smaller doses were less effective, but daily doses as low as 1–5 mg. exerted a recognizable action. Twice daily doses were probably more effective than once daily ones. The higher doses (above 50 mg. daily) produced considerable falls in the microfilaria count, but even these did not remove all microfilariae from the blood or prevent their increase again after the treatment had ceased. Similar results were obtained when five rats were given an intensive dosage of 1,100 mg./kg. during nine hours. Numerous living microfilariae were found in the pleural cavities at post mortem. No attempt was made in these preliminary studies accurately to count the number of adult worms found at autopsy, and the females were accorded more attention than the males; consequently the results shown in Table I are qualitative rather than quantitative. They are enough, however, to show that hetrazan in these dosages had exerted little action upon most of the adult worms. Only 1 out of 160 recorded male worms was dead, and only 19 of about 500 females (worms from dead rats are excluded). Further work on adult worms will be described below.

Analysis of the Action of Hetrazan on Microfilariae

Action in vitro.—Microfilariae from citrated heart blood were washed in saline by repeated centrifugation (the upper layers of sediment contained the microfilariae); the microfilariae, so isolated, were washed in Tyrode solution and resuspended in this fluid at a concentration of 20,000 to 100,000 per c.c., so that inoculation of 0.005 c.c. of the suspension into 0.1 c.c. of medium in an observation vessel involved the addition of 100 to 500 organisms. The use of distilled water or of detergents as haemolytic agents in the isolation of microfilariae was distinctly harmful to them. The observation vessels were blown from Pyrex glass tubing of 4 to 5 mm. bore to a shape somewhat like a Carrel flask, being hemispherical, with flat bases and open oblique side arms; they were wide enough for 0.1 c.c. of liquid to lie on the floor of the vessel without the formation of a bubble against the roof. Fluids were introduced through Wright's pipettes of various sizes, and the side arms were closed with sealing-wax. Sterile precautions were used throughout. Microfilariae in the observation vessels were studied through an inverted microscope. Several fluids were examined to determine how long microfilariae would live in them. Only those which contained serum were satisfactory, but no distinction could be made between the sera of cotton rat, white rat, rabbit, and horse. The best diluent was Tyrode solution, and the best serum/diluent ratio was 20/80. White rat serum and Tyrode solution were subsequently used in all experiments.

The results of three experiments are summarized in Table II. Briefly, hetrazan in high concentration (4 mg. per ml.) had no effect on the survival of microfilariae *in vitro*, and sera taken from rats ½–2 hours after they had received the maximum tolerated dose of hetrazan were also ineffective. It was concluded that the *in vivo* action of hetrazan against microfilariae is not due to any direct lethal action of the drug itself or of any hypothetical derivative in the body.

Speed of action of hetrazan.—The preliminary studies showed that many microfilariae disappeared from the blood very rapidly after the intraperitoneal injection of hetrazan, e.g., Fig. 6A. Passage of drug from the peritoneal cavity into the blood occupies an unknown amount of time and therefore further investigations were made

TABLE II

SURVIVAL OF MICROFILARIAE *in vitro* AT 37° C. IN THE PRESENCE OF HETRAZAN

Drug or serum*	Concentration	Survival of microfilariae in hours		
		Experiment		
		1	2	3
Hetrazan	4.0 mg. per ml.			40
	2.0 ,, ,,			40
	1.5 ,, ,,			40
	1.0 ,, ,,		50	
	0.5 ,, ,,		70	40
	0.25 ,, ,,		50	40
	0.1 ,, ,,	35	30	
	0.05 ,, ,,	50	50	
Serum after ½ hour ..	50 per cent			64
	20 ,, ,,		25	40
	10 ,, ,,		25	40
Serum after 1 hour ..	50 ,, ,,			64
	20 ,, ,,		25	23
	10 ,, ,,		25	87
Serum after 2 hours	20 ,, ,,	25	25	
	10 ,, ,,	50	25	
Controls	No drug	30	30	52

* The sera were taken from white rats which were killed at ½-2 hours after they had been treated intraperitoneally with hetrazan 500 mg. per kg.

by intravenous injection. The rat was anaesthetized by tribromoethanol or pentobarbitone and injections were made into the jugular vein which was exposed in the neck, the dose being 60 mg. per kg. in a volume of 0.25 c.c. A typical result is given in Fig. 1, which shows that hetrazan acts with remarkable rapidity, removing 80 per

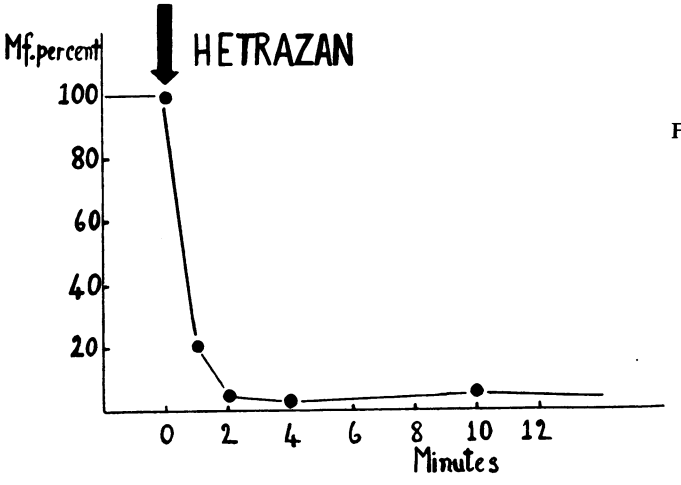


FIG. 1.—Disappearance of microfilariae from the blood when hetrazan (60 mg. per kg.) is injected intravenously. The number of microfilariae is given as a percentage of the number initially present, viz. 258 per 10 cu.mm.

cent of the microfilariae from the blood within one minute and 95 per cent within two minutes. In spite of this extremely rapid onset, however, the fall in the microfilaria count does not proceed to zero, but continues fluctuating about the 5 per cent level, with a slight tendency to rise again; the reason for this persistence and subsequent rise again is probably the migration of fresh microfilariae from the pleural cavities into the blood.

Hetrazan in absence of living adult worms.—An experiment was planned to exclude any disturbing influence due to the presence of living adult worms and to see whether the action of hetrazan was affected by the age of the microfilariae. A group of infected rats was treated with 500 mg. pentostam per kg. daily for five days in order to kill the adult worms. These rats were taken in pairs after various intervals, hetrazan was injected intraperitoneally, and the microfilaria count was studied in blood from the tail. Forty-eight or seventy-two hours later the rats were killed and autopsied. The curves showing the response of the microfilariae in these rats to hetrazan are shown in Fig. 2; for those of two normal control rats see Fig. 6A. The microfilariae in rats 1386 and 1388 were 47 days older than those in rats 1373 and 1374. Actually the shapes of the curves in the two Figures are not significantly different; and although the blood of rats 1386 and 1388 was made microfilariae-free by a single injection of hetrazan, while that of 1373 and 1374 required several injections, this difference may be due to the fewer numbers in the blood when hetrazan treatment was first begun. In the two rats (1373 and 1374) treated with hetrazan four days after pentostam the pleural cavities contained many microfilariae (3,000–6,000 per 10 cu.mm.) although the adult worms had all been killed by the pentostam; the tail blood in both rats was free from microfilariae, but the blood of the right ventricle contained 0 and 2 Mf. per 10 cu.mm. respectively,

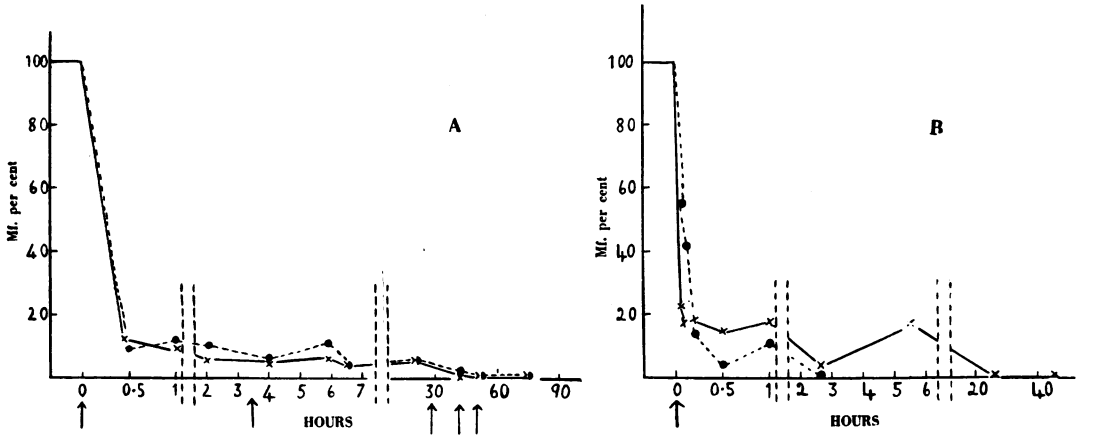


FIG. 2.—Effect of hetrazan upon microfilariae in rats previously treated with pentostam. The vertical scale shows the number of microfilariae in the blood as a percentage of its level before treatment. The arrows show the times when hetrazan (250 mg./kg.) was injected intraperitoneally. (A) Rats treated 4 days after the last dose of pentostam. Rat 1373, 306 microfilariae per 10 cu.mm. (continuous line); rat 1374, 684 Mf. per 10 cu.mm. (broken line). (B) Rats treated 51 days after pentostam. Rat 1386, 28 Mf. per 10 cu.mm. (continuous line); rat 1388, 58 Mf. per 10 cu.mm. (broken line).

while that of the left ventricle contained 4 and 15 Mf. per 10 cu.mm. In the two rats (1386 and 1388) treated with hetrazan 51 days after pentostam the pleural fluid contained only a few microfilariae (about 20 per 10 cu.mm.) and the blood from both tail and heart contained none. These findings show that in rats whose blood has been freed from microfilariae by hetrazan there still persists a store of living parasites in the pleural cavity, whence they migrate through the lung into the blood stream (as can be seen in histological sections of the lung, and as is further illustrated by the microfilaria content of blood in the left ventricle being higher than that of the right). Microfilariae from this store in the pleura constantly replenish those in the circulating blood ; so that although hetrazan rapidly *decimates* the microfilariae in the blood, repeated dosage is necessary to *abolish* them completely, and they often return quite speedily.

Distribution of microfilariae in tissues before and after hetrazan

Different organs were examined histologically in order to find what had happened to the microfilariae which had disappeared from the blood ; for microfilariae are comparatively large parasites which can be traced with relative ease. For this purpose rats were treated with hetrazan intraperitoneally (250 mg. per kg.) and killed at various intervals. Blood (10 cu.mm.) was taken from the tail before and after treatment for counting the microfilariae. When the thorax was opened, the adult worms were removed from the pleurae and a drop of pleural fluid was examined for microfilariae, which were always very numerous, e.g., 5–10 thousand per 10 cu.mm. A small incision was then made into each ventricle in turn and a haemoglobin pipette inserted in order to remove 10 cu.mm. blood for counting ; this procedure may have allowed contamination from the pleural fluid and so yielded too high counts. In later experiments a ligature was placed round the base of the heart, which was then excised and washed in saline ; blood was then obtained from each ventricle by piercing the wall with a fine Pasteur pipette. The organs were removed, weighed, and fixed in formol saline. The volume of the lungs was estimated by immersing them (before fixation) in a measuring cylinder of Ringer's fluid. The organs were sectioned and stained with haemotoxylin and eosin. The sections were searched systematically at a magnification of approximately 700 and the number of microfilariae was counted. Usually about 30–40 sq.mm. of section were examined, but in important cases this area was more than doubled. The count included only the microfilariae which were in the capillaries (or tissues) of the organs ; microfilariae lying in the blood vessels (other than minute venules) were excluded and considered to belong to the number present in the circulating blood. If two fragments of microfilariae were seen near each other they were interpreted as parts of a single worm and counted as one. In the tissues a few worms were sometimes difficult to identify ; they formed only a small minority and were counted as half. When microfilariae are counted in a section the count consists mostly of portions of microfilariae which lie parallel or oblique to the plane of the section ; microfilariae which are perpendicular to the section cannot be easily distinguished from nuclei and they were not included in the counts of either organs or blood clot. This technique assumes that microfilariae are orientated at random in blood clot and tissues ; this is true of most tissues, but it might not hold for striated muscle or for the medulla of the kidney, where most of the capillaries are parallel to each other ; such tissues were sectioned in the plane of the capillaries. The area of the section was determined by projecting its magnified image on to squared paper, and measuring the area of the latter by weighing. In this way the number of microfilariae per unit area was obtained. This was converted into microfilariae per unit volume by the following empirical method.

Blood was taken from the heart of a heavily infected rat and placed in a small waxed paper box about 1 cm. cubed ; 20 cu.mm. samples were taken to form thick films for counting the microfilariae. The remainder of the blood was allowed to clot slightly and it was then fixed in corrosive sublimate plus acetic acid, or formol saline. The clot was embedded in paraffin and cut for histological section on four different parallel and vertical planes. The sections were stained with haematoxylin and eosin and the microfilariae were counted as in the sections of tissue above.

In the first experiment the counts of the blood films showed 1,605, 1,630, 1,716, and 1,780 Mf. per 20 cu.mm. ; average 1,680. The microfilariae counted in four cross sections were: 52, 50, 54, and 71 Mf. per 31 sq.mm. ; average 56.7 Mf. In the second experiment, the average number of microfilariae per 20 cu.mm. blood was 1,070, and the average number in a section of clot was 229 per 100 sq.mm. If x Mf. per 100 sq.mm. corresponds to y Mf. per 100 cu.mm. Then $a\ x$ Mf. per 100 sq.mm. corresponds to $a^{\frac{3}{2}}\ y$ Mf. per 100 cu.mm. Then in the first experiment

a Mf. per 100 sq.mm. corresponds to 3.29 $a^{\frac{3}{2}}$ Mf. per 100 cu.mm. and in the second experiment

a Mf. per 100 sq.mm. corresponds to 4.90 $a^{\frac{3}{2}}$ Mf. per 100 cu.mm. The average of these two estimates is that

a Mf. per 100 sq.mm. corresponds to 4.20 $a^{\frac{3}{2}}$ Mf. per 100 cu.mm. and this conversion figure has been used in the ensuing calculations.

The distribution of microfilariae in the different organs before and after hetrazan treatment is shown in Table III, which summarizes the findings in 4 untreated rats and in 21 treated rats. The microfilariae in the organs are given as the number per 100 *square* millimetres of a section ; this can be converted into microfilariae per 100 *cubic* millimetres by using the conversion factor just described. The data will be considered from several aspects.

(*a*) *The distribution of microfilariae in untreated rats.*—The figures for the four untreated rats have been analysed further in Table IV, which gives the volumes of the different organs, the absolute number of microfilariae in each, and the percentage of the total number of the body in each site. (Although the microfilariae in the pleural cavity are very numerous (5–10 thousand per cu.mm.) they have not been included in these calculations, since they are considered as not yet having entered into the general circulation.) The estimates of the different quantities are only approximate, but they are sufficient for the present purpose. The average figures for untreated rats show that about 63 per cent of the total microfilariae are in the blood, 30 per cent are in the lungs, 2.6 per cent each in the liver and kidney, and insignificant amounts in the other organs (except possibly muscle and skin). The numbers present in the skeletal muscles (average 1.3 per cent) and in the skin (? none) are unfortunately a little uncertain. The density of microfilariae in these organs is very low, so that they are rarely found in sections and cannot be accurately estimated ; on the other hand, the volumes of the two organs are so large (approximately 45 and 18 per cent of the total volume of the rat (Donaldson, 1924)) that even a low density might account for a large absolute content of parasites. Although no microfilariae were observed in the skin capillaries, presumably some must occur in this site to permit transmission by the insect vector. The microfilarial content of the

TABLE

THE DISTRIBUTION OF MICROFILARIAE IN RATS AFTER TREATMENT

Each column refers to one rat. The figures in parentheses under the

Hours after hetrazan :	Control	Control	Control	Control	0.33	1.0	1.0	1.25	1.5	1.75	2.25
Rat No. :	1438	1585	2270	F22	1616	F4	1684	1685	1639	1645	1644
Mf. per 10 *cu.mm. blood*											
In life, before hetrazan	920	535	753	2,800	187	663	474	200	1,965	1,520	144
In life, after hetrazan ..					29		85	94	178	465	19
					(0.33)		(1.0)	(1.0)	(1.3)	(1.25)	(2.2
At death, L. ventricle ..	1,840		2,280	4,200	51	34	100	188	267	9,330	225
R. ventricle ..			700	4,560	57	59	510	80	509	940	25
Mf. per 100 *sq.mm. tissue*											
Liver :											
Biopsy before hetrazan					1.4		44	22			
Autopsy	20.4	25.4	4.7	115.2	18.7	494	200.3	205	361	267	59.2
Bone marrow	0		11.1	39.4	30.5	0	0		0	6.4	5.4
Spleen	6.1	1.3	16.8	96	0	53.7		5.4	62.5	71	0
Lung	730	225	204	1,700	16.7	121	46		92		19.2
Kidney	82.5	25.7	113	71	4.1	5.7	3.6	14.6	51.4	49	7.6
Heart	12.3	9.1	39.1	117	5.3	7.6	0		31.8		0
Skeletal muscle	0	4.5	0	2.9	1.5	0	1.9		1.1		
Brain			11.1		2.1		11.6		6.7		0
Skin			0	0			0		0		

III

WITH HETRAZAN, 250 MG. PER KG. INTRAPERITONEALLY

" microfilariae in blood " figures are the hours after treatment.

4.3	4.45	5.45	5.6	6.17	17.55	23.55	24.0	27.45	47.25	47.6	48.2	72.6	72.6
1650	1665	F7	F5	1668	1631	1680	1651	1682	1687	1705	1708	1676	1679
58	1,861	263	120	591	2,651	927	93	211	618	1,600	320	800	255
7	351	368		71	274	181	12	23	84	435	58	33	40
(1.9)	(1.2)	(2.9)		(1.2)	(0.3)	(1.2)	(2)	(1)	(1.1)	(1)	(1.1)	(5.6)	(5.6)
5	555			58	411	260	12	43	78	50	44	22	30
(4.3)	(4.4)			(6.1)	(17.5)	(23.5)	(22)	(27.4)	(30)	(29.6)	(30)	(23)	(23)
									90	21	8	19	9
									(47.2)	(47.6)	(48.2)	(72.6)	(72.6)
24	5,310	1,480	99	38	3,796	5,421	130	100	652	520	117	49	400
20	920	589	62	37	765	1,443	161	120	90	32	6	224	190
18.2	241	40	44	88.8	55	9.8	4.1	2.4	5.1	6	0	11	5.7
	0				4.2								
0	134			12.4	47.5								
	160			16	198								
0	51	6.6		15.1	103	26.6	0	0	3.4	7.9	3.7	15.8	13.4
	13.9			7.5	48.8				16.2				
				0	3.8				0				
	2.2			0	20.4				3.3				
	0				0				0				

TABLE IV

ANALYSIS OF THE DISTRIBUTION OF MICROFILARIAE IN FOUR UNTREATED RATS AND IN FOUR RATS TREATED WITH HETRAZAN; BASED ON TABLE III

Organ	Control rats						Treated rats					
	Rat No.	Volume c.c. per 100 g.	Mf. per 100 sq. mm.	Total Mf. × 10^3	Per cent of total Mf.		Rat No.	Volume c.c. per 100 g.	Mf. per 100 sq.mm.	Total Mf. × 10^3	Per cent of total Mf.	
					Per rat	Average					Per rat	Average
Blood ..	1438	(6.3)	—	870	59.7	62.9	1616	(6.3)		27	49.5	19.7
	1585		—	270	66.0		F4			37	2.1	
	2270		—	460	76.0		1684			53.5	13.3	
	F22		—	2,300	50.0		1639			215	14.0	
Liver ..	1438	4.1	20.4	15.4	1.06	2.56	1616	6.4	18.7	21.5	39.5	74.5
	1585	3.2	25.4	17.3	4.23		F4	3.5	494	1,620	91.7	
	2270	3.7	4.7	1.59	0.26		1684	2.85	200	336	83.2	
	F22	(3.7)	115.2	218	4.70		1639	4.5	361	1,288	83.8	
Marrow	1438	(?0.2)	0	0	0	0.032	1616	(?0.2)	30.5	1.5	2.75	0.69
	1585		—	—	—		F4		0	0	0	
	2270		11.1	0.31	0.05		1684		0	0	0	
	F22		39.4	2.1	0.045		1639		0	0	0	
Spleen ..	1438	0.16	6.1	0.10	0.007	0.029	1616	0.15	0	0	0	0.069
	1585	0.15	1.25	0.0094	0.002		F4	0.09	53.7	1.49	0.084	
	2270	0.14	16.8	0.40	0.066		1684	—	—	—	—	
	F22	(0.15)	96	5.90	0.13		1639	0.09	62.5	1.87	0.122	
Lung ..	1438	0.66	730	543	37.4	30.2	1616	0.73	16.7	2.26	4.13	3.77
	1585	0.69	225	98	24.0		F4	1.9	121	111	6.28	
	2270	0.76	204	92.5	15.3		1684	0.9	46	11.8	2.93	
	F22	(0.70)	1,700	2,050	44.3		1639	0.72	92	26.6	1.74	
Kidney	1438	0.91	82.5	28.6	1.96	2.60	1616	0.79	4.1	0.27	0.49	0.18
	1585	0.73	25.7	3.98	0.97		F4	1.07	5.7	0.62	0.035	
	2270	0.84	113	42.5	7.0		1684	0.8	3.55	0.23	0.057	
	F22	(0.81)	71	20.4	0.44		1639	0.76	51.4	1.17	0.13	
Heart ..	1438	(0.5)	12.3	0.9	0.06	0.15	1616	(0.5)	5.3	0.10	0.18	0.050
	1585		9.1	0.57	0.14		F4		7.6	0.18	0.01	
	2270		39.1	5.13	0.85		1684		0	0	0	
	F22		117	26.0	0.56		1639		31.8	1.51	0.099	
Muscle	1438	(45)	0	0	0	1.32	1616	(45)	1.5	1.7	3.12	0.95
	1585		5.0	21	5.1		F4		0	0	0	
	2270		0	0	0		1684		1.9	2.5	0.62	
	F22		2.9	9.20	0.20		1639		1.1	1.08	0.07	
Brain ..	1438	(1.7)	—	—	—	0.43	1616	(1.7)	2.06	0.21	0.38	0.15
	1585		—	—	—		F4		—	—	—	
	2270		11.1	2.64	0.43		1684		11.6	0.28	0.069	
	F22		—	—	—		1639		6.7	0.124	0.008	
Skin ..	1438	(18)	—	—	—	0	1616	(18)	—	—	—	0
	1585		—	—	—		F4		—	—	—	
	2270		0	0	0		1684		0	0	0	
	F22		0	0	0		1639		0	0	0	
Total	1438			1,458.0			1616			54.54		
	1585			410.9			F4			1,770.3		
	2270			607.0			1684			404.3		
	F22			4,631.6			1639			1,535.4		

lungs may not be a reliable index to what happens in other animals infected with filariasis; in cotton rats, the microfilariae migrate from the pleural cavity through the lung to reach the blood, and this migration may make the density in the lung higher than would otherwise be the case; all the same it is noteworthy that the lungs contain five times as many microfilariae as the other viscera put together, and that the density is especially great.

(*b*) *Distribution of microfilariae immediately after hetrazan.*—After the intraperitoneal injection of hetrazan (250 mg. per kg.) the concentration of microfilariae in the blood falls (as expected) but the concentration in the liver increases greatly.* The figures may be analysed in various ways, attention being limited first to the rats killed within five hours, i.e., to the *immediate* effects of hetrazan. The distribution of microfilariae between the different parts of the body was calculated for four rats killed about one hour after treatment with hetrazan (Table IV). Only about 20 per cent of the microfilariae were in the blood, but about 75 per cent were in the liver. The percentages in the lungs and kidney fell considerably (to 4 and 0.2 per cent respectively), parallel with that in the blood. About 1 per cent were in skeletal muscle, but none was observed in the skin. Attention may next be directed to the three rats (161, 1684, 1685) in which the liver was sampled before and after hetrazan. (These rats were anaesthetized with pentobarbitone, a small piece of liver was removed by biopsy, hetrazan was then injected, and 20–75 minutes later the rat was killed with chloroform and a full autopsy was made.) In rat 1616, the microfilariae of the blood fell from 187 to 29 per 10 cu.mm., i.e., 158 Mf. per 10 cu.mm. blood disappeared or 100,000 Mf. altogether (assuming 6.3 c.c. blood per 100 g. rat). The liver content rose from 1.35 Mf. to 18.7 Mf. per 100 sq.mm. Since the liver weighed 6.4 g. per 100 g. at autopsy, the total number of microfilariae

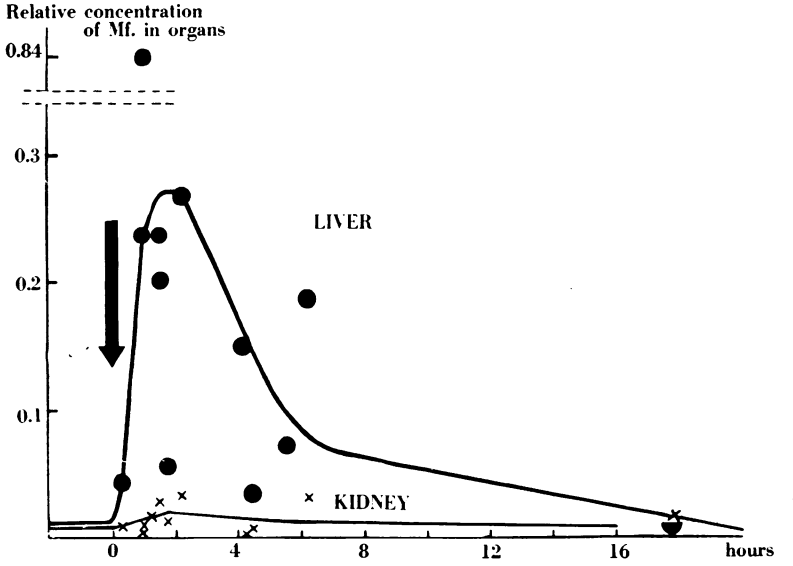


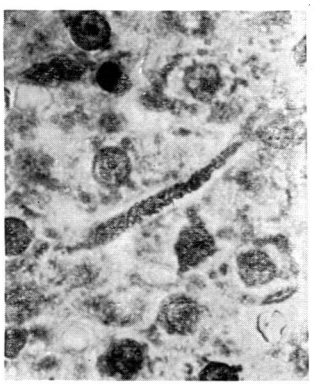
FIG. 3.—The relative concentration of microfilariae in the liver, compared with that in the blood, at different times after the intraperitoneal injection of hetrazan (250 mg./kg.); the relative concentration in a neutral organ such as the kidney is shown for comparison. The figures show the ratio between the number of microfilariae per 100 sq. mm. in the liver or kidney respectively and the number per 100 cu.mm. in the blood. Each spot indicates the value obtained for the liver from one rat and each cross represents a similar value for the kidney.

*In one rat (F7) hetrazan failed to reduce the microfilariae of the blood, and the relative number of microfilariae in the liver did not increase, an accidental but striking illustration of the inverse relationship between these two quantities.

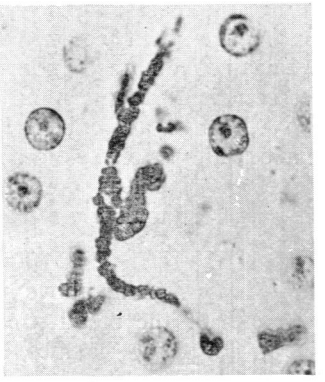
appearing in the liver was 21,000. Similar calculations show that in rat 1684 (liver, 2.85 g. per 100 g.) 240,000 Mf. disappeared from the blood and 300,000 Mf. appeared in the liver; and that in rat 1685 (liver 3.34 g. per 100 g.) 67,000 Mf. disappeared from the blood and 400,000 Mf. appeared in the liver. These estimates are not very accurate, but they are sufficient to show that the number of additional microfilariae appearing in the liver can account for those which have disappeared from the blood.

(*c*) *The concentration of microfilariae in the liver relative to the time after hetrazan.*—Fig. 3 illustrates the relation between time after the administration of hetrazan and the number of microfilariae in the liver, relative to those in the blood at death (average of tail blood and blood of R. ventricle). Before hetrazan is given the relative content of microfilariae in the liver is very low, but very soon after the injection of hetrazan it rises rapidly. According to the curves for the disappearance of microfilariae from the blood (Figs. 1 and 6), the microfilariae must be collected in the liver during the first few minutes after the hetrazan reaches the blood stream. The figures for the various animals vary greatly, so that it is difficult to say when the number in the liver begins to decline; but it is possible that this begins as early as 4–6 hours. By 18–23 hours the relative number of microfilariae in the liver has fallen back to its original level, where it continues for the rest of the period of observation (to 72 hours). Since there is no corresponding increase in the microfilaria content of the blood at 18 hours and afterwards, it must be presumed that the microfilariae disappearing from the liver after 6 hours have been destroyed. Meanwhile, in a presumably passive organ such as the kidney, the microfilaria-content, relative to the blood, remains substantially unchanged after treatment with hetrazan. The small increase in the ratio (kidney/blood) is probably due to a time-lag before the concentration of microfilariae in the kidney adjusts itself to the diminished concentration in the blood.

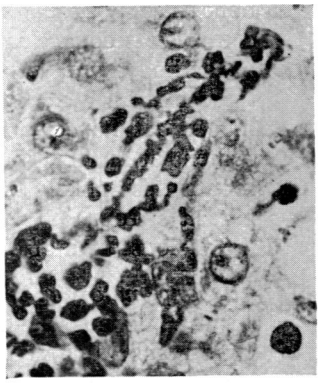
(*d*) *Histological appearances.*—Histological study gives further information about the fate of the microfilariae in the liver (see Fig. 4). In untreated rats, the microfilariae lay in the sinusoids of the liver without any cellular reaction around them; they filled the sinusoid completely, but presumably during life they were able to work their way through the vessel into the veins. Twenty minutes after hetrazan had been given (as judged by rat No. 1391) the number of microfilariae present had increased greatly; they lay in the sinusoids which were closed around them. Presumably they were held in some way, but there was no clear histological evidence as to the mechanism involved. One hour after treatment many of the microfilariae still lay in the sinusoids, without cellular reaction; but other microfilariae (varying from 10–60 per cent of the total number) were partly or completely surrounded by phagocytes with oval or kidney-shaped nuclei and extensive cytoplasm stretched out along the surface of the parasite; presumably they were Kupffer cells; there were also a few polymorphs present. In some of the rats killed at 4–6 hours the phagocytes round microfilariae formed an extensive cluster, displacing the liver cells; there were also similar foci in which microfilariae could not be discovered; it was not clear whether all these foci were due to microfilariae or whether some of them might have been focal necroses due to low grade bacterial infection; in some of the microfilariae the nuclei were disarranged, suggesting that they were being destroyed.



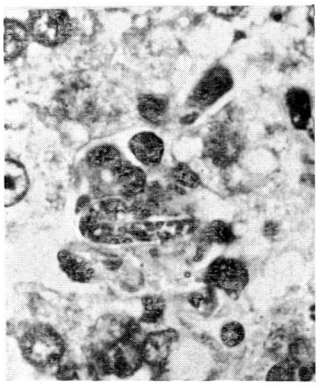
(A) Liver of untreated rat; no phagocytes.



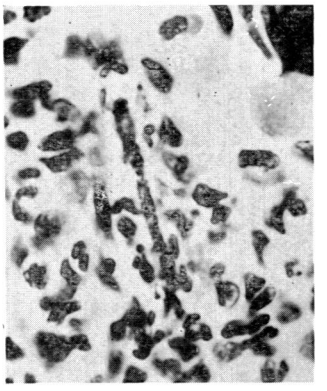
(B) Liver, 1 hr. 5 min. after hetrazan; some phagocytes near the microfilaria.



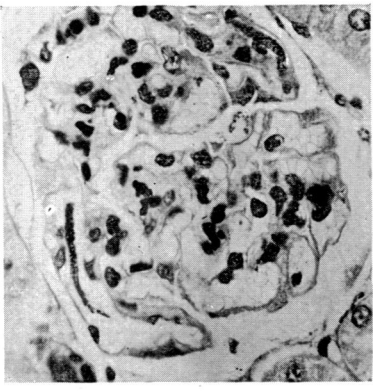
(C) Liver, 5 hr. 40 min. after hetrazan; many phagocytes present.



(D) Liver, 17 hr. after hetrazan; most of the microfilariae have been destroyed by this time.



(E) Spleen, 4.5 hr. after hetrazan; the microfilaria is attacked by phagocytes.



(F) Kidney, untreated rat × 700.

FIG. 4.—Microfilariae in the capillaries of rats, fixed in formol-saline and stained haematoxylin and eosin; × 1,250.

After 28 hours, some of the few microfilariae present were surrounded by phagocytes and showed evidence of disintegration ; others lay free in the sinusoids (perhaps these were new microfilariae which had entered the blood stream subsequent to the treatment with hetrazan).

(*e*) *Conclusions.*—From all this work, the hypothesis is put forward that hetrazan removes microfilariae from the blood stream by modifying them in some way so that they are held in the liver and there destroyed by phagocytes of the reticulo-endothelial system. Thus hetrazan would act like an opsonin. In rats, most of the reticulo-endothelial system is collected in the liver (6 per cent of body weight), while the spleen (0.3 per cent of body weight) contains a much smaller proportion and the

bone marrow probably contains even less. Table IV shows that the spleen of three control rats contained on the average about 0.03 per cent of the total microfilariae of the body, whereas after hetrazan it contained about 0.07 per cent. According to these figures, the spleen does contribute to the removal of microfilariae from the blood, but its contribution is so small as to be insignificant. The microfilaria content of marrow is irregular but almost always low ; in view of its small volume in rats, it is unlikely that it makes any significant contribution to the removal of these parasites from the blood. In larger animals or in man the relative importance of the various parts of the reticulo-endothelial system might well be quite different.

Confirmatory experiments.—In view of the above hypothesis, consideration may be given to microfilariae lying outside the blood stream. In these rats there are large numbers of microfilariae in the pleural cavities, but prolonged treatment with hetrazan has no appreciable effect on their motility or on their relation to the cells of the pleural exudate. Two infected rats were treated by intrapleural injection of hetrazan twice daily (250 mg. per kg.) for 4 doses. The microfilariae in the blood stream were reduced to less than 5 per cent of the initial count, as usual. When the rats were killed on the third day, the microfilariae (and the adult worms) in the pleura were found to be apparently unaffected. These findings confirmed that hetrazan has no direct toxic effect on the microfilariae ; apparently the phagocytosis of microfilariae after hetrazan involves only the fixed macrophages of the liver, etc., and not phagocytes free in the body fluids.

Systematic experiments with hetrazan plus microfilariae plus leucocytes *in vitro* have not yet been undertaken. Attempts were made to blockade the reticulo-endothelial system by the intravenous injection of colloidal copper combined with splenectomy. When hetrazan was injected an hour later, the microfilariae disappeared from the blood at approximately the usual rate. In other experiments microfilariae were injected intravenously into mice, with or without previous incubation with hetrazan ; in both cases 95 per cent of the microfilariae disappeared from the circulating blood in a few minutes, so that this procedure seemed unsuitable for demonstrating the action of hetrazan.

Hetrazan and cataphoresis.—Simple experiments were made on the electrical charges of microfilariae, with and without hetrazan. The observation vessel was a piece of glass tubing 5 cm. long with a right-angle bend at each end opening to a short vertical tube about 1 cm. long, the tube being flattened on the side opposite to the vertical extensions. A current of 4 m.amp. at 15V. was passed through this cell between potassium chloride-agar electrodes. The microfilariae were suspended in the standard serum-Tyrode mixture and left in the cell for ten minutes, in which time they had settled on the bottom ; they were then free from the effects of circulation in the suspending fluid. Normally, they were orientated at random, but after the current had flowed for ten minutes they all pointed head towards the anode ; this orientation was independent of fluid movements. Reversal of the current completely reversed the orientation. As the microfilariae continually wriggled spontaneously, this orientation resulted in their gradual migration towards the anode, at a rate of about 0.2 mm. per min. The addition of hetrazan (up to 0.1 per cent) to the suspending fluid produced no change in the orientation or rate of migration of

the microfilariae. There was thus no evidence that hetrazan causes any gross alteration in their surface potentials.

Attempts to inhibit the action of hetrazan.—Attempts were made to inhibit the *in vivo* action of hetrazan by the previous administration of some compound with a similar chemical structure, just as *p*-aminobenzoic acid resembles and inhibits sulphanilamide. Rats were inoculated subcutaneously with diethylurea (400 mg. per kg.), nicotinamide (2 × 100 mg. per kg.), nicotinic acid (100–250 mg. per kg.), niketham ide (2 × 100 mg. per kg.), quinine (200 mg. per kg.), or miracil D (200 mg. per kg.). An hour later, the standard dose of hetrazan (250 mg. per kg.) was injected intraperitoneally and microfilariae counts were made at frequent intervals. In three control rats, the lowest microfilaria counts (expressed as a percentage of the original count) during the next five hours were 7.7, 4.5, and 4.9.

After diethyl urea the count fell to 2 per cent;
after nicotinamide, to 9.7, 0, and 6 per cent;
after nicotinic acid, to 10, 14, and 23 per cent;
after niketham ide, to 14, 7.5, and 7.3 per cent;
after quinine, to 6.7 per cent;
and after miracil D, to 10 per cent.

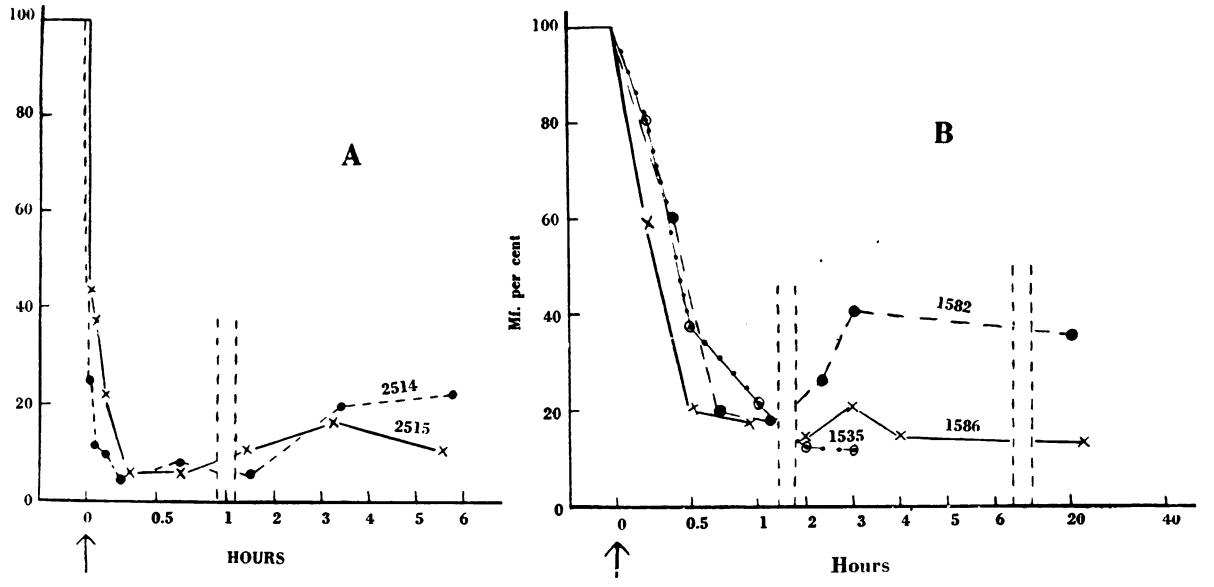


FIG. 5.—The influence of nicotinic acid on the effect of hetrazan upon microfilariae in rats. The vertical scale shows the number of microfilariae in the blood as a percentage of its level before treatment. The arrow shows the time when hetrazan (250 mg. per kg.) was injected i.p. (A) Two control rats. Rat 2514, 2,456 Mf. per 10 cu.mm. Rat 2515, 1,265 Mf. per 10 cu.mm. (B) Three rats, previously treated with nicotinic acid. Rat 1535, 3,085 Mf. per 10 cu.mm.; nicotinic acid, 250 mg./kg. i p. 55 min. earlier; toxic. Rat 1582, 338 Mf. per 10 cu.mm.; nicotinic acid, 150 mg./kg. i.p. 20 min. earlier; toxic. Rat 1586, 510 Mf. per 10 cu.mm.; nicotinic acid, 100 mg./kg. s.c. 30 and 2 min. earlier.

None of these compounds appeared to have any interfering action on hetrazan except perhaps nicotinic acid. The curves for the microfilaria counts in the rats treated with hetrazan, with or without previous nicotinic acid, are shown in Fig. 5. Repetition of this experiment using a small dose (50 mg. per kg.) of hetrazan showed no interference by nicotinic acid.

Action of Hetrazan on Adult Worms

In the preliminary *in vivo* studies, it was found that hetrazan had little obvious effect on the adult worms; this was investigated further. Sixteen rats were given 250 mg. hetrazan per kg. twice daily for 14 days and were killed 8 or 28 days after the last dose. The worms present in the pleural cavities were counted and examined for any sign of being attacked by phagocytes. Seven untreated rats served as controls. The results are shown in Table V. Out of 431 female worms found in the

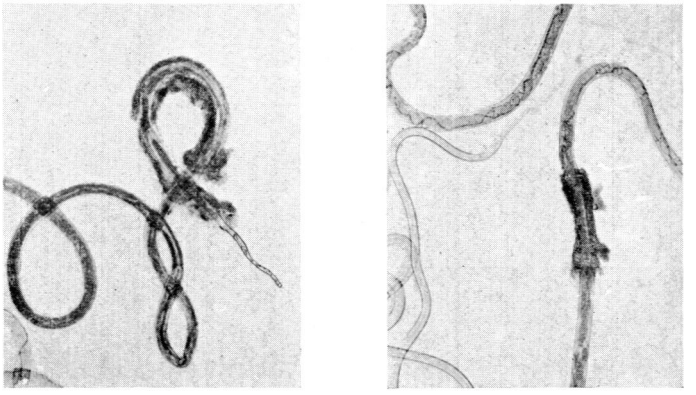
TABLE V

EFFECT OF INTENSIVE TREATMENT WITH HETRAZAN ON ADULT WORMS *in vivo*

250 mg. per kg. twice daily for 28 doses, intraperitoneally. Each horizontal line refers to one rat

	Microfilariae per cu.mm. of tail blood						Post mortem findings				
	Before first dose	Days after first dose					Days since last dose	Days since infection	Female worms		Male worms
		1	7	14	23	40			No. dead/total No.	No. living with phagocytes	No. dead/total No.
TREATED RATS	70	1	0	0			8	83	0/45	0	0/17
	42	0.2	0.3	0.1			8	83	0/41	0	0/39
	20	0.8	0.1	0			8	83	0/40	0	0/40
	116	0.3	0.1	0			8	83	1/13	0	1/13
	2	0	0	0			8	83	1/5	0	0/4
	2	0	0		0.2		8	76	2/4	0	—
	8	0.2	0		2		8	76	2/3	0	0/3
	1	0.1	0		8		8	76	2/9	0	0/5
	40	0.6	0	0.1	5	2	28	103	4/24	0	0/8
	32	0.2	0.1	0	5	0	28	91	4/17	0	1/3
	6	0	0	0	0	6	28	91	1/10	2	1/7
	16	0.1	0	0	0.8	8	28	91	3/11	0	0/4
	15	1	0	0.3	5	30	28	123	16/157	0	0/13
	36	0	0		4		28	96	3/8	0	0/6
	96	0.3	0		24		28	96	0/31	2	0/7
	98	0	0		10		28	96	1/13	0	1/1
									40/431	4	4/170
CONTROL UNTREATED RATS	36							61	0/110	1	—
	78							104	1/220	0	1/151
	28							104	3/134	0	0/91
	2							70	0/14	0	0/17
	236							76	0/6	0	0/10
	170							76	0/5	0	0/1
	170							87	0/29	0	0/14
								87	0/6	1	0/7
									4/524	2	1/291

16 treated rats, 40 (9.3 per cent) were recently dead, while out of 524 female worms in the 8 control rats, 4 (0.76 per cent) were recently dead. (The remains of immature dead worms are excluded, since these had died before the drug was given.) The difference between the two groups is statistically significant ($6.5 \times$ S.D), but it is doubtful whether much weight should be attached to it, especially as 16 out of the 40 dead worms occurred in a single rat which had been infected rather longer than the others. Accordingly the lethal action of hetrazan on adult female worms seems to be slight. The male worms are equally insusceptible. As regards phagocytes, four of the female worms from the treated rats and two from the controls had clumps of phagocytes adherent to them, as is shown in Fig. 6. These phagocytes were not



A B

FIG. 6.—Phagocytes adherent to worms from rats treated with hetrazan, 250 mg./kg. i.p. twice daily. (A) Fore part of worm, 2 days after last dose, × 15. (B) Worm from rat killed 8 days after last dose, × 15.

located around a body aperture, e.g., vulva or anus. Since this occurred equally rarely in both groups, there is no evidence that hetrazan promotes destruction of the adult worms by phagocytosis (as occurs with the microfilariae).

Very prolonged treatment.—Two groups of infected rats were treated with hetrazan by mouth for 108 days. For this purpose the hetrazan was dissolved in their drinking water. The estimated daily intake of hetrazan was about 10 mg. and 100 mg. per kg. in the two groups respectively (Table VI). This treatment caused most of the microfilariae to disappear from the blood, but it did not remove them completely even after 108 days. On the other hand, there is no evidence that the microfilariae became hetrazan-resistant during this period. When the drug was stopped, the microfilariae gradually increased in number again. At autopsy most of the female worms were alive; the few dead ones discovered were not more numerous than one would expect to find in untreated rats which had been infected for so long.

Adult worms in vitro.—Adult worms were removed from rats with sterile precautions and placed in Carrel flasks at 37° C. in a medium consisting of two parts Tyrode to one part serum (horse or cotton rat). They lived well and if the medium

TABLE VI

EFFECT OF PROLONGED TREATMENT WITH HETRAZAN ON ADULT WORMS AND MICROFILARIAE *in vivo*

Hetrazan was put into the drinking water.

Dose schedule	Microfilariae per cu.mm. of tail blood											Post mortem findings		
	Start	Days after first dose										Days since last dose	Days since infection	Female worms
		1	5	12	20	28	108	122	132	216	309			No.dead/ total No.
c.10 mg./kg. daily														
108 days	89	13	2	2	0.2	0	0.3	0	5	10		128	339	—
108 ,,	112	65	3	5	0	2		5	10	75	9	201	412	0/20
101 ,,	10	5	0	1	0	0.2	Rat died					0	204	0/many
98 ,,	33	0.6	4	0.7	0.3	0	Rat died					0	201	0/many*
c.100mg./kg. daily														
108 days	124	4	2	0.1	0.2	0.4		0.2	5			26	229	0/20
108 ,,	436	2	1	0.5	0	0.2	0.6	10	60	175		162	365	5/15
108 ,,	135	6	46	0	0	0.4	0.5	5	5	40	76	201	404	3/30
23 ,,	374	42	2	0.7	1	Rat died						0	118	0/many

* No microfilariae in pleural fluid; uteri seemed empty.

was changed at 3–5 day intervals controls survived for more than 17 days. (In peptone water and broth they lived for 3–5 days.) When neutralized hetrazan citrate was added the longest periods of survival were: in 500 mg. per 100 ml., 3 days; in 200 mg., 5 days; and in 100 mg., 14 days. In 33 per cent serum from a white rat treated one and a half hours previously with 250 mg. hetrazan per kg. the worms lived up to 13 days. Since these concentrations of drug are much higher than those encountered in the body, it is concluded that neither hetrazan nor any hypothetical derivative of it in the body has a direct lethal action on the adult worms.

Action on Worms in Mosquito

Investigations were made to see whether hetrazan has any action upon the filarial worm of dogs, *Dirofilaria repens*, developing in mosquitoes (*Anopheles maculipennis atroparvus*). The strain of *D. repens* had been kindly supplied by Mlle. van Hoof, of the Prince Leopold Institute for Tropical Medicine, Antwerp. Hetrazan was given to the mosquitoes by putting it in the glucose solution on which they feed, according to the technique described by Terzian (1947) for testing antimalarials. Preliminary experiments showed that glucose containing 1 per cent hetrazan was well tolerated. Further experiments showed that *D. repens* developed normally in *Anopheles* fed throughout on glucose containing 1 or 0.5 per cent hetrazan; infective forms were found in the proboscis after 14 days in good health and in approximately the same numbers as in the control mosquitoes. Apparently hetrazan has no effect on the development of this species of filaria in the insect vector.

Action on Infective Larvae and Immature Worms

Cotton rats were exposed to mites (*L. bacoti*) infected with *Litomosoides*, according to the standard technique described by Hawking and Sewell (1948). (For this purpose the rats are placed for 14 days in tanks containing the mites, and then are stored for seven weeks until the worms become mature and microfilariae appear in the blood.) Some of the rats in each tank were kept as controls, and some were treated with drugs during exposure or during the development of the worms according to the schedules shown in Table VII. Neostam (an antimonial) and the cyanine dye, No. 863* (Welch, Peters, Bueding, Valk, and Higashi, 1947), are two drugs

TABLE VII

THE EFFECT OF DRUGS UPON THE INITIATION AND DEVELOPMENT OF FILARIAL INFECTIONS

The rats were exposed to infective mites for 14 days.

Treatment			No. of rats developing microfilariae/No. of rats exposed to infection	
Start	Frequency	Drug and dose, mg. per kg.	Treated	Controls
4 days after first exposure; continued till 2 days after last	Daily for 12 days	Hetrazan, 500 Cyanine No. 863, 0.1 Neostam, 160	0/2 3/3 0/4	3/3 4/4 7/7
6 days after last exposure	Twice daily for 5.5 days	Hetrazan, 500	2/2	3/3
14 days after last exposure	Daily for 6 days	Hetrazan, 250 Cyanine No. 863, 0.1	4/9 † 2/2	8/8 7/7
28 days after last exposure	Daily for 6 days	Cyanine No. 863, 0.1	0/2	18/18

† The 5 rats without microfilariae harboured female worms but no males.

which are known to kill the adult worms *in vivo*. The large doses of hetrazan killed some of the rats in the initial group, and these have been excluded from the Table. Hetrazan given during exposure to the mites prevented microfilariae appearing in two rats, which suggests that it acts upon the infective larvae ; but only two rats were studied and unfortunately they were not killed for examination at the end to see whether sterile worms were present. Hetrazan given at 6 or 14 days after exposure to mites failed to prevent microfilariae appearing in 6 of 11 rats. The five rats without microfilariae were found at autopsy to harbour only female worms with numerous undeveloped and degenerating ova in the uteri. Apparently hetrazan does not prevent the female worms developing, once the infective larvae have settled down in the host, but it may have a selective action upon the development of the males.

The cyanine dye did not protect rats when administered during exposure or from fourteen days after exposure, but it protected two rats when given twenty-eight days after exposure ; apparently it acts on the mature or almost mature worms but not

*1'ethyl-3,6-dimethyl-2-phenyl-4-pyrimido-2'-cyanine chloride.

on the immature ones. Neostam protected rats when given during exposure ; apparently it acts on the infective larvae as well as on the adult worms. The number of rats used in some of these groups is too small for the results to be conclusive. Further investigations are planned.

Discussion

The antifilarial action of hetrazan may best be discussed separately for each stage in the life history of the worm. Briefly, it has a pronounced action *in vivo* on the microfilariae, only a slight action on the adult worms, and no action on the developmental stages in the mosquito, while its action on the infective forms and early stages in the rat is not quite certain.

Microfilariae.—As shown above hetrazan has no significant lethal action *in vitro*, but *in vivo* it causes them to disappear rapidly from the blood. This is associated with the accumulation of microfilariae in the liver (and to a minor degree, in the spleen and marrow) where they are destroyed by phagocytes. Normally, microfilariae are so well adapted to their habitat in the vertebrate host that they apparently excite no significant humoral or cellular action. In some way hetrazan alters this harmonious adaptation, and they are then removed from the blood by the normal mechanisms for dealing with foreign bodies in this fluid. The alteration produced by hetrazan is still obscure. In some respects it resembles the effect of specific immune serum upon bacteria, promoting their phagocytosis, i.e., opsonization ; but probably this analogy should not be pressed far. Such a mode of action is almost unknown in chemotherapy with the one exception of suramin (Bayer 205, Antrypol). According to Jancsó and Jancsó (1934) this compound acts by sensitizing trypanosomes so that they are destroyed by phagocytes of the reticulo-endothelial system ; if this system is " blocked " by splenectomy and the intravenous injection of colloidal copper, the efficacy of suramin is much diminished. As mentioned above no great success attended attempts to repeat this type of experiment with hetrazan and rats infected with filariasis.

Hewitt *et al.* (1947) state that hetrazan has an *in vitro* action upon the microfilariae of *Folyella dolichoptera* of frogs, causing first contraction and then immobility. Nothing like this was seen with the microfilariae of *Litomosoides* during the present work, and it seems unlikely that this is the way hetrazan destroys microfilariae *in vivo*.

Adult worms.—According to the results described above, hetrazan has no discernible effect upon adult worms *in vitro* ; *in vivo* an intensive course of hetrazan killed 9.3 per cent of 431 female worms, while in the control untreated animals only 0.76 per cent of 524 female worms were dead. The difference between these two groups is statistically significant, so that it must be concluded that hetrazan probably does have a slight lethal action on adult females *in vivo* ; but the action is so small compared with that of antimonials, arsenicals, and cyanine compounds that it is unlikely to be of any practical importance. According to our experience with other compounds, the male worms are less susceptible to chemotherapy than the females, and this holds for hetrazan also within its very limited range. In the group of rats under intensive treatment (Table V), only 2.3 per cent of 170 male worms were dead, as compared with 9.3 per cent of 431 females. No evidence could be obtained that hetrazan promoted phagocytic action. Hewitt *et al.* (1947) report the finding of

many dead worms in rats which had been treated with hetrazan and then held for over 70 days before autopsy. They worked with wild rats which had been infected for an unknown period before capture, and the dead worms which they found had probably been killed by the immunity of the host as often happens in ageing infections. These authors also treated dogs infected with *Dirofilaria immitis*; living worms were present in all the 10 dogs in which adult worms were found at all; apparently hetrazan is not very effective in killing the adult worms of *D. immitis* either.

Infective larvae and immature worms.—Unfortunately our experimental results on this subject are too few to be conclusive. They suggest that hetrazan destroys the infective larvae when they invade the rat, but that it has much less action on the immature worms after they have established themselves in the rat for a few days. Our results also suggest that immature males are more susceptible than immature females, which is in contrast to our experience with adult worms. Further investigation is planned.

Development in the mosquito.—Apparently hetrazan has no action in preventing the development of *Dirofilaria repens* of the dog in mosquitoes. If hetrazan acts (as suggested) by promoting phagocytosis, this is what would have been expected.

SUMMARY

1. The antifilarial action of hetrazan (1-diethylcarbamyl-4-methylpiperazine) was investigated in cotton rats infected with *Litomosoides carinii*. It is very effective in removing microfilariae from the blood; this action is very rapid, 80 per cent of the microfilariae being removed in one minute.

2. The age of the microfilariae did not affect their response. In cotton rats there is a store of microfilariae in the pleural cavity, which are not affected by hetrazan; these migrate through the lung into the blood and replenish the number of microfilariae in the blood when it has been reduced by hetrazan. This explains why single doses of hetrazan do not clear the blood completely.

3. Hetrazan has no lethal action on microfilariae *in vitro* at 37° C., and serum from an animal treated with hetrazan is also inactive.

4. A study was made of the distribution of microfilariae throughout the body. In untreated cotton rats about 63 per cent are in the circulating blood, 30 per cent in the capillaries of the lungs, and 2.6 per cent each in those of the liver and of the kidney. Soon after the administration of hetrazan, almost 75 per cent of the microfilariae are in the capillaries of the liver, while only about 20 per cent are in the blood and 4 per cent in the capillaries of the lung. The number of microfilariae accumulating in the liver is enough to account for those which disappear from the blood. Later (after 6–18 hours) the number of microfilariae in the liver diminishes, apparently because the parasites have been destroyed. Histologically the microfilariae collected in the liver capillaries are soon surrounded by phagocytes which apparently destroy them. It is concluded that hetrazan acts on the microfilariae *in vivo* by modifying them so that they are held in the liver and there destroyed by phagocytes of the reticulo-endothelial system; thus to some extent hetrazan resembles an

opsonin. Trifling numbers of microfilariae are destroyed in the spleen. Microfilariae which lie *outside* the blood stream, e.g., in the pleural cavities, are not destroyed by hetrazan.

5. Hetrazan does not affect the surface electrical potentials of microfilariae. It was not possible to inhibit the action of hetrazan by compounds of similar chemical structure, apart from doubtful inhibition observed after the previous administration of nicotinic acid.

6. On adult worms *in vivo* hetrazan has only an insignificant lethal effect. It does not promote the phagocytosis of adult worms. Prolonged exposure (108 days) to hetrazan did not destroy the adult worms; and there was no evidence that the microfilariae became resistant to hetrazan during this period. Adult worms *in vitro* are not affected by hetrazan or by its metabolic derivatives.

7. Worms (*Dirofilaria repens*) developing in the mosquito are not affected by hetrazan. Some evidence was obtained that infective larvae (*L. carinii*) are destroyed by hetrazan. Immature female worms are not affected *in vivo*, but there may be a selective action on the immature male worms. A cyanine dye acted on the mature or almost mature worms but not on the earlier stages. Neostam destroyed worms in any of the stages in the rat.

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