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THE MODE OF ACTION OF DIETHYLCARBAMAZINE INVESTIGATED WITH ^{14}C-LABELLED DRUG

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Investigation of the metabolism and excretion of isotopically labelled diethylcarbamazine (Bangham, 1955) showed that this drug is rapidly metabolized and excreted in the urine after intravenous or oral administration. Ten to 15% of the dose is excreted as unchanged drug, 10–15% as diethylcarbamylpiperazine, 2–5% as methylpiperazine, and 1–6% as piperazine. The remainder of the dose—approximately 60%—is excreted as a compound which has been isolated in crude form but not yet identified.

The present paper describes the distribution of the drug in the organs and tissues of the cotton rat (*Sigmodon*) and of the hooded rat (*Rattus*), the uptake of drug by microfilariae and adult worms, and the action of the drug on microfilariae in the first few minutes after intravenous injection.

METHODS

Estimation of Radioactivity

The methods for estimation of total radioactivity in solids and liquids, separation and estimation of individual metabolites, chromatography counting techniques and limits were as described by Bangham (1955).

Microfilaria Counts

The cotton rats which were used weighed 150–300 g. and were reared in this Institute and infected here with *Litomosoides carinii* (Hawking and Sewell, 1948). Only animals with heavy infections were used.

A blood sample was drawn into a calibrated 10 mm.^{3} siliconed dry pipette, diluted by taking up a similar volume of 1% potassium citrate, and discharged on to a grease-free slide marked with two parallel lines 2 cm. apart. The pipette was rinsed again with about 10 mm.^{3} potassium citrate which was spread with the sample over the area—2 cm. × slide width (2.5 cm.). The slides were allowed to dry flat before being stained. Great care was needed during staining to avoid lifting and detachment of the edge of the film. Slides were usually stained with Giemsa, since this method was less likely to detach fragments from the film than staining with haemalum.

The microfilariae were counted under a ×10 objective and ×10 eyepiece by examining the width of the film (2 cm.) at 5 constant distances apart, i.e., the same geometric areas on each different film.

Demonstration of the Rate of Disappearance of Circulating Microfilariae after Intravenous Administration of the Drug

Blood-sampling Methods.—In order to investigate closely the disappearance in the first few minutes, a technique for rapid repetitive and reproducible blood-sampling was needed. Initially samples were taken from the left external jugular vein at the root of the neck. A small hole was torn in the vein wall through which just enough blood for each sample was allowed to leak. The hole could easily be closed by firm pressure with a pledget of cotton wool and opened again by a light scratch.

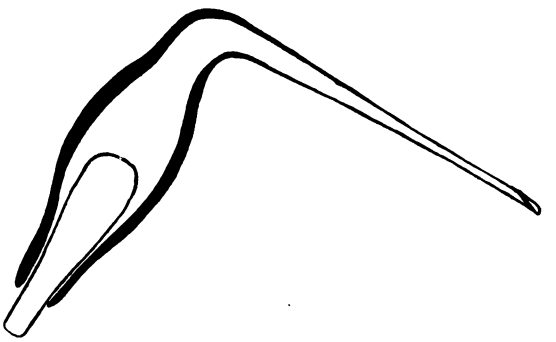

FIG. 1.—Polythene valve-cannula.

Later, a better technique for rapid sampling was used for experiments with radioactive drug. The technique (Bangham, A. D., personal communication) consisted in controlling the outflow from an artery by means of a small push valve in a polythene cannula.

One end of a small polythene tube was drawn down to fit closely round a short length of tapering glass rod. The polythene tube was cut short so that the glass projected 3–5 mm. when the valve was closed. If the projecting tip was pushed in, the valve was opened (Fig. 1). The other end of the tube was drawn out and cut obliquely for easy insertion into an artery. The cannula had a capacity of about 0.02 ml.

When the cannula was tied into the carotid artery, the arterial blood pressure forced the glass valve out

into the taper, and no blood could escape. The valve end of the cannula was then fixed into a base-plate carrying the rat so that the glass tip hung down vertically. A blood sample could then be taken by momentarily pushing the glass valve upwards.

Samples were taken on to perspex discs, 11 cm. in diameter, resting on three needle points set in a disc rotated by a 1 rev./min. Sangamo electric motor (Fig. 2). A second disc (hanging free of the needles and underneath the sample disc) was attached to the central movable core of an electromagnetic coil. On activation of the coil this second disc lifted the sample disc free of the needles. By adjusting the height of the coil core when raised, the sample disc could be made to impinge on the projecting glass valve of the cannula, so releasing a drop of blood. The size of the drop depended on the activation-time of the coil. A Palmer clock was used to control the timing and duration of the activation of the coil. With the cannula used, samples were released every 6 sec. and an activation time of about 1 sec. released samples measuring not more than 0.05 ml. blood.

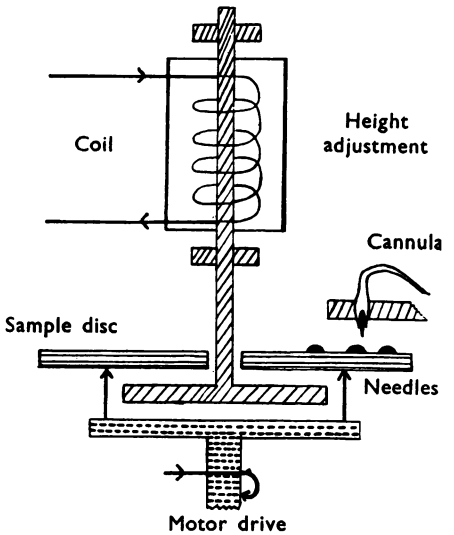

FIG. 2.—Rapid blood-sampling apparatus.

Procedure.—An infected cotton rat was anaesthetized with 1.5 ml. of 20% urethane intraperitoneally, and the cannula was tied into a carotid artery. Heparin (0.2 ml. of 1% soln.) was injected through the cannula, which was then fixed in the base plate. A syringe carrying the dose was connected in readiness to a fine needle in a superficial femoral vein. The base plate was adjusted over the sampling disc and 4 samples were taken at 10-sec. intervals to provide a base line for microfilaria counts. The dose of radioactive diethylcarbamazine (16.5 mg./kg. base in 0.25 ml. = 1.5 μc.) was injected during 10–18 sec. Just before the injection the sample timing was changed to once every 6 sec. and 10 samples were taken. The time was then readjusted to once every 10 sec. for 12 samples. Finally a sample was taken every 30 sec., the disc being rotated by hand. As each sample disc was loaded it was taken off and placed on ice, and another clean one was substituted.

The volume of blood removed 3 min. after injection of the dose was thus not more than 22 × 0.05 ml. = 1.1 ml. This was approximately 10–20% of the blood volume of a 200 g. rat. Over the first few minutes it provided a reproducible set of samples on which microfilaria counts, radioactivity assays, and chromatograms could be done.

For comparative purposes blood was assumed to have a constant carbon content, and counts on 1 cm.^{2} infinitely thick samples of barium carbonate are reported on this assumption. A chloroform extract of the blood samples was made directly they were taken from ice, and the extract was run as a chromatogram the same evening.

This technique differed from the blood sampling method first described in three ways : arterial blood was taken ; more samples were removed more rapidly ; and the animal had been injected with heparin. Heparin was necessary to prevent the slow clotting that occurred in the cannula despite heavy siliconing of the glass valve rod.

Uptake of Diethylcarbamazine by Tissues in the Rat in vivo

Rats of 150–200 g. weight were given an intravenous dose of piperazine ring-labelled drug and killed at various time intervals by a blow on the head. The organs were dissected and weighed after light blotting on filter paper. No correction was made for blood in the specimen, but care was taken to avoid any adipose tissue. The organ, or a piece of the tissue, was then combusted and the $BaCO_3$ counted. Rats were killed at 5 min., 10 min., 1 hr., and 6 hr. after an intravenous dose of approximately 25 mg./kg. base (7 μc./dose).

Uptake of Drug by Blood Cells in vitro

Fresh whole blood from normal hooded and cotton rats was heparinized (0.02 μg./ml.) and incubated at 37° C. with 5 or 50 μg. of labelled drug/ml. At 5, 15, 60, and 180 min. a sample was removed, and was centrifuged for 15 min. in a haematocrit tube ; measured volumes of plasma, red cells, and buffy coat were taken for assay of radioactivity by combustion. To obtain a greater number of white cells, two normal hooded rats were inoculated subcutaneously with 0.2 ml. of a yeast-saline suspension a week previously ; a sterile abscess developed at the injection site, and the white cell count of the blood was approximately twice the normal. No correction was made for plasma included among the cells, and they were not washed by resuspension because of the apparently easy diffusability of the drug.

No significant concentration by white or red cells was observed ; the drug seemed to diffuse evenly and rapidly among all cells.

Uptake of Drug by Worms of L. carinii in vitro

Adult *L. carinii* worms were collected from cotton rats killed with coal gas. Only undamaged lively worms were used after they had been separated into batches of males and females. They were incubated in 10 ml. of a 2/3 Ringer-glucose 1/3 horse serum medium containing 36, 58, or 65 μg. drug/ml. at 37° C. After 1 hr., when they were still wriggling vigorously, they were lifted out with a needle and rinsed for about 10 sec. in four amounts of 8 ml. of the drug-free medium, being dried carefully on filter paper between each rinse. After weighing they were either ground up with sand and carrier and the drug assayed as the reineckate, or they were dried in a vacuum desiccator for 24 hr., weighed, and combusted, and the activity assayed as $BaCO_3$.

Uptake by Microfilariae of Dirofilaria immitis

Microfilariae of this species were used in preference to those of *Litomosoides* because of their larger size and of the ready availability of blood from an infected dog. It is known that diethylcarbamazine is active against these microfilariae.

The dog was anaesthetized with intravenous pentobarbitone sodium and 24 ml. blood withdrawn into heparin. This blood was divided into four batches of 6 ml., each of which was injected into 75 ml. cold distilled water in a large conical centrifuge tube, and mixed by gentle shaking. Lysis of red cells was stopped after 5 min. by the addition of an appropriate volume of a concentrated saline solution to bring the salt concentration back to 0.9%. The microfilariae were then spun down gently for 10 min., and the supernatant saline was removed. The microfilariae were transferred with 10 ml. of Ringer-glucose solution into a smaller tube and centrifuged again to a total volume of 1.0 ml. To this was added 0.02 ml. of a stock solution of labelled drug to give a concentration of approximately 200 μg. base/ml. After 1 hr. in the incubator at 37° C. with occasional mixing, 9 ml. of Ringer-glucose was added and the solution centrifuged for 5 min. at 1,000 rev./min. Nine ml. of the supernatant fluid was replaced with fresh Ringer-glucose and, after mixing the solution, centrifuged again. After another similar wash, as much as possible of the supernatant fluid was removed and the sludge of microfilariae was transferred to a platinum combustion boat for weighing. The boat was then placed in a vacuum desiccator over P_2O_5 overnight and weighed again before combustion of its contents. Known amounts of carrier succinic acid were added to some small samples to provide adequate $BaCO_3$ for weighing and counting. No attempt was made to correct for washing solution included in the microfilarial sludge, but an assay was made on an aliquot of the last washing solution used (Table II). The three rinsings with Ringer-glucose eliminated a large amount of blood cell debris, and examination under the microscope showed little such material among the microfilariae.

Effect of Metabolites of Diethylcarbamazine on the Activity of Microfilariae in vitro

A suspension of microfilariae of *L. carinii* in 1/3 serum 2/3 saline was divided between two tubes, and test material to give a concentration of 1% was added to one tube. Both tubes were kept in a water bath at 37° C. and small amounts of the suspension were removed alternately from each tube and examined under the microscope every 2 min. An arbitrary scale of 4 points was used to indicate the activity of the microfilariae observed over 20 min.

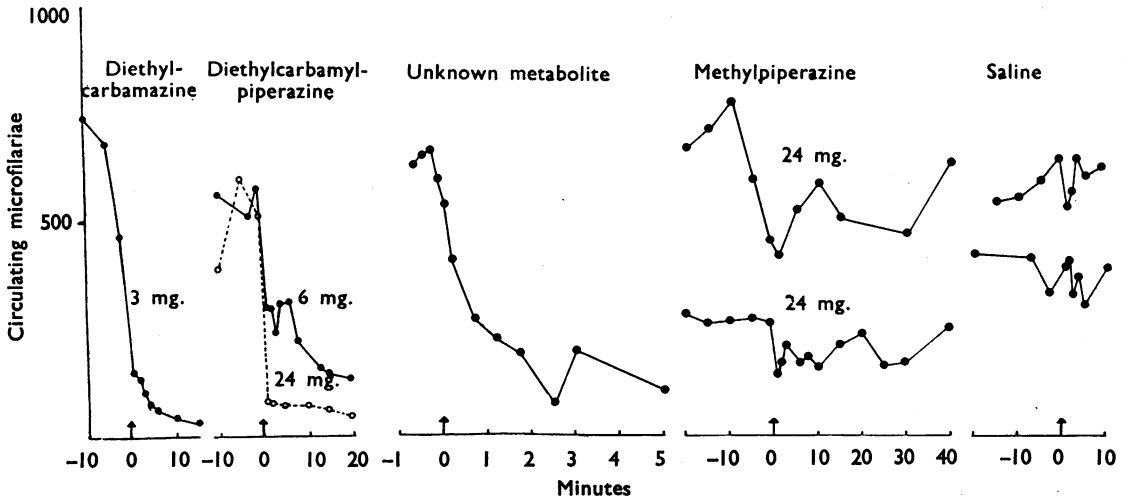

FIG. 3.—Effect of i.v. injection in 0.5 ml. saline of (1) diethylcarbamazine, (2) diethylcarbamylpiperazine, (3) unknown metabolite, (4) methylpiperazine, and (5) control injections of saline, on the number of circulating microfilariae in cotton rats (250 g.) infected with *Litomosoides carinii*.

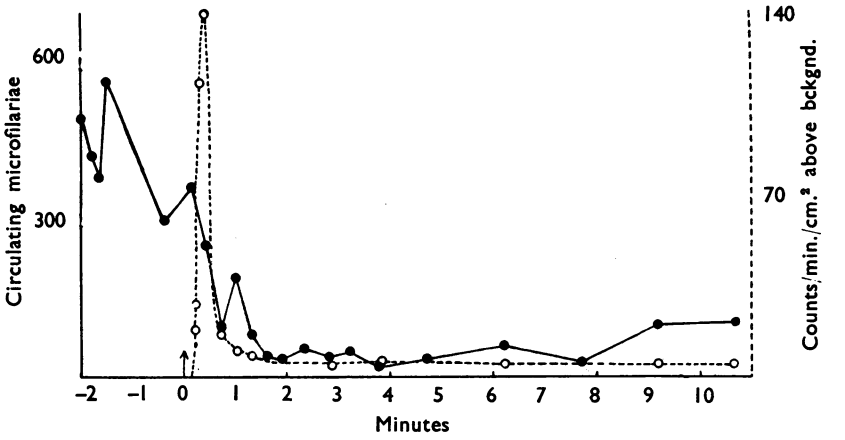

FIG. 4.—Correlation of drop in circulating microfilariae with the injection of ^{14}C-labelled diethylcarbamazine.

RESULTS

The degree of fluctuation in the number of circulating microfilariae of *L. carinii* that may normally occur is shown in Fig. 3 in the experiments where methylpiperazine or saline was injected. No immediate or sustained drop in numbers occurred after these injections. On the other hand, diethylcarbamazine caused the microfilariae to disappear rapidly from the circulating blood. Diethylcarbamylpiperazine exerted a similar effect when a higher dose was given. The result of one experiment, in which the breakdown product of the unknown metabolite was injected, showed that this substance was also active. Fig. 4 shows the fall in microfilariae in relation to the concentration of the circulating drug. Chromatograms of blood extracts suggested the presence of a substance with R_F 0.5, which is the same value as that of the unknown metabolite. Its identity cannot be established because a method of crystallization after isolation of the metabolite has not yet been found.

The average uptake of diethylcarbamazine by the tissues of the rat, determined 5, 10, 60, and 360 min. after an intravenous dose, was respectively 1.3, 0.77, 0.67, and 0.30% of the dose/g. tissue. There was no significant concentration of the drug by liver, common bile duct, muscle, kidney, spleen, lung, adrenal, skin, gut, vertebral column, testis, fat, or blood cells. None of these had an uptake of more than four times the average; only the kidney maintained a higher average than the other tissues, presumably because of the presence of urine.

No significant concentration by leucocytes or erythrocytes of *Rattus* or *Sigmodon* was observed *in vitro*; the drug seemed to diffuse evenly and rapidly into all cells.

The absorption of diethylcarbamazine by adult *L. carinii in vitro* is shown in Table I. Female worms varied markedly in size in different batches —presumably according to the age of the infection—so individual batches are reported. The

TABLE I

UPTAKE OF DIETHYLCARBAMAZINE BY ADULT WORMS OF *LITOMOSOIDES CARINII IN VITRO*

Worm		Wt. (mg.)		Incubation		Uptake of Drug by Filarial Worms	
Sex	No.	Wet	Dry	Time	Medium	μg./g. Wet	μg./g. Dry
					μg./ml.		
♀♀	61	100	14·8	1 hr.	36	60 } 55·5	401 } 300
♀♀		87	14·9	1 ,,	36	57	299
♂♀	57	17·5	4·5	1 ,,	36	21	81
♀		80·1	13·5	3 ,,	36	38	250
♀♀	100	98	16·5	1 ,,	58	28 } 35	175 } 216
♀♀	54	84·5	14·0	1 ,,	58	42	258
♂♀		16·4	4·8	1 ,,	58	19	64
♀		108·1	17·5	3 ,,	58	9	54
♀	69	228	37·6	1 ,,	65	59	355
♂		17	4·9	1 ,,	65	54	274

difference in size may well account for the variation in the uptake of drug by male and female worms after 1 hr. The significance of the relative decrease in amount of drug found in female worms after 3 hr. is not known. When the results were expressed as μg./g. wet weight there was no significant concentration of drug by these worms above the level of the medium.

Table II shows the uptake of diethylcarbamazine by microfilariae of *D. immitis* incubated with the drug for 1 hr. *in vitro*. The concentration of drug in the organisms was only about one-third of that in the medium.

TABLE II

UPTAKE IN 1 HR. OF DIETHYLCARBAMAZINE BY MICROFILARIAE OF *DIROFILARIA IMMITIS IN VITRO*

Microfilariae		Medium	Uptake of Drug by Microfilariae		Concn. of Drug/ml. of Last Wash
Wet Wt.	Dry Wt.		μg./g. Wet	μg./g. Dry	
19·0 mg.	1·8 mg.	210 μg./ml.	66	595	1·0 μg./ml.
40·6 ,,	2·7 ,,	210 ,,	57	635	
69·3 ,,	6·2 ,,	200 ,,	64·6	712	1·65 ,,
—	1·3 ,,	200 ,,	—	654	— ,,

Neither diethylcarbamazine nor any of its four metabolites was found to have a significant effect upon the activity of *L. carinii* microfilariae *in vitro*.

DISCUSSION

The Possible Mode of Action of Diethylcarbamazine

It is well known that diethylcarbamazine rapidly reduces the number of circulating microfilariae of *W. bancrofti, W. malayi,* and *Loa loa,* and that those of *O. volvulus* die rapidly during treatment (Hawking, 1950; Hawking, Sewell, and Thurston, 1950). Its effect upon the adult worms of these species is still under dispute. Yet, so far, very little is known of the mode of action of this drug upon microfilariae, or that of the simpler polyvalent piperazine salts which are used for treating the larger helminths, *Ascaris,* whip worm, *Ancylostoma braziliensis,* etc. (Hawking, 1955).

It was soon established that diethylcarbamazine had little or no apparent effect upon microfilariae *in vitro* (Hawking *et al.,* 1950). These organisms can exist in a 1% solution of the drug in plasma, serum, or saline for many hours with no effect on their motility or histological structure. On injecting the drug intravenously in the cotton rat infected with *Litomosoides carinii,* however, the number of circulating microfilariae is reduced by about 80% in 2–3 min. Hawking *et al.* (1950) have demonstrated by histological methods that microfilariae are collected in the liver, which in the cotton rat contains most of the fixed reticuloendothelial system. Within a few hours the organisms are surrounded by phagocytes, and a few hours later they are obviously disintegrating.

This contrast of the rapidity of effect of diethylcarbamazine on microfilariae in the experimental animal, and *in vitro* respectively, is without parallel among chemotherapeutic agents.

There are three ways of interpreting how this drug exerts its effect. The drug may modify the surface of the microfilaria and act as an "opsonin"; it may be metabolized to form a substance, stable or unstable, which is directly lethal to the organism; or the drug may affect the host animal in such a way that it can "filter off" and phagocytose the organism.

The conception that the drug acts as an "opsonin" was put forward by Hawking (1950), and it is the only suggestion so far made in the literature. Several non-specific substances are known to act like opsonins for certain bacteria in biological systems *in vitro* (Topley and Wilson, 1946). Tannic acid, gallotannic acid, chrome alum and ferric salts, protamine, and globin all act in such a way. The metallic tanning agents and the strongly basic proteins, protamine and globin, are thought to combine with the carboxyl groups of bacterial proteins. These substances, like natural "opsonins," increase phagocytosis of bacteria *in vitro,* increase cohesiveness between particles, and lower the electric charge on the bacterial surface as determined by cell electrophoresis.

It would be difficult to demonstrate any one of these properties on microfilariae *in vitro.* Microfilariae normally exhibit a certain cohesiveness which does not apparently increase on exposure to the drug, while their large size (8×100 μ) and vigorous movement make it difficult to demonstrate phagocytosis outside the confines of a capillary. The interpretation of movement in an electrophoretic cell also becomes uncertain. This theory is therefore difficult to assess by these direct criteria. However, the experiments described above indicate that the clearance of microfilariae from the circulating blood by the drug is nearly as rapid as the clearance of foreign particles—such as dead bacteria or carbon black—introduced into the blood stream. Such a rapid clearing suggests a physical "filtering" by fixed macrophages or capillary endothelium. It is possible that within a minute of intravenous injection of drug the microfilariae, which are normally freely circulating, become denatured or modified so as to be recognized and treated as foreign particles. Microfilariae removed from the blood stream a few minutes after treatment with the drug still wriggle vigorously and look normal both before and after staining. They are not abnormally cohesive and they are not surrounded by phagocytes. Moreover, these few organisms which survive in the blood stream often do not disappear for some days despite repeated doses of drug.

Although little is known about the quantitative uptake of natural opsonins by bacteria, there is certainly no marked concentration of drug by microfilariae or adult worms (the females of which contain large numbers of immature microfilariae).

It would be of interest to see if this drug exerts any opsonizing effect upon bacteria in the biological systems already described in the literature.

The second possibility that a metabolite of diethylcarbamazine might be directly toxic to microfilariae seems unlikely when their rate of clearance from the blood stream is appreciated. Although the rapidity with which the labelled carbon of the methyl group appears as expired carbon dioxide suggests that metabolism is remarkably prompt (Bangham, 1955), the amount metabolized in the first two minutes is so small that any compound so formed would be present in about one-thousandth of the concentration of the drug administered. Moreover, chromatograms of blood and urine indicate that the unidentified metabolite is the first one to appear, and the evidence from hydrolysis experiments suggests that this metabolite still retains this methyl group. The simple demethylated compound diethylcarbamylpiperazine appears later in the urine and is known to be biologically less active.

It seems from the work of Hewitt and his co-workers (1947, 1948) that the piperazine ring is necessary for microfilaricidal activity, but that the alkyl radical itself is not essential in position 4. An increase in length of this radical results in diminished activity and increased toxicity. On the other hand, a carbethoxy group in position 1 with a number of different alkyl radicals in position 4 gave rise to a series of active compounds. The only compounds which lacked the carbethoxy group and which showed marked activity against microfilariae possessed an ethyl-, di*iso*propyl-, dimethyl-, or diethyl-carbamyl group in position 1. It is interesting, therefore, that the unknown metabolite showed an infra-red spectrum not unlike that of the 1-ethoxycarbonyl-4-methylpiperazine examined, and that this unknown metabolite is active *in vivo*. However, even if these other side chains were converted to a carbethoxy group, or if these side chains and the carbethoxy group were all metabolized to a common substance which was biologically active, the same anomalous situation arises, since neither this unknown metabolite nor any of the other metabolites have any apparent effect upon microfilariae *in vitro*. It is still possible to postulate an unstable intermediary metabolite being directly lethal to these organisms (this would be difficult to prove), but neither blood nor plasma incubated with drug, nor blood taken from a rat a few minutes after a dose, has any visible effect upon them.

Thirdly, it is possible that the drug has some effect upon the tissues of the host which enables it to remove microfilariae from the circulation. Several simple di-substituted piperazine compounds are in fact now recognized as having potent pharmacological properties (Roth, 1954; Jordan, Wheeler, Foye, and Orth, 1954). A number of such compounds examined by Roth (1954) showed a diversity of pressor blocking effects as well as antihistamine and local anaesthetic activity. At least two antihistamines used clinically (cyclizine hydrochloride (Norton, Colville, Light, Wnuck, Fanelli, and de Beer, 1954) and chlorcyclizine hydrochloride (Packman, Rossi, and Harrison, 1953)) contain a methylpiperazine group. The benzhydrylpiperazine group is also antihistaminic (Albro, Baltzly, and Phillips, 1949). Methylphenylpiperazine has been shown to possess marked adrenaline-blocking and reversing activity as well as local anaesthetic and other effects (Roth, 1954). Diethylcarbamazine itself, however, has few immediate pharmacological effects beyond producing a transient hyperpnoea, so it seems unlikely that any major circulatory change could account for the biological action. It is conceivable that diethylcarbamazine modified macrophages or the capillary endothelium in some way —possibly analogous to the action of the anionic detergent polyoxyethylene ethers on macrophages and tubercle bacilli (Mackaness, 1954)—so as to facilitate the trapping and phagocytosis of microfilariae. This also would be extremely difficult to prove. None of the tissues or organs of the rat examined showed any preferential uptake of the drug *in vivo* in the experiments described above.

The results of this investigation with isotopically labelled drug thus yield evidence that could support the theory that diethylcarbamazine has an opsonin-like action upon microfilariae, although direct proof of this mode of action must probably await a fuller understanding of the general anthelminthic properties of piperazines.

Summary

1. The clearance of microfilariae of *Litomosoides carinii* from the circulating blood by diethylcarbamazine and its metabolites in the first few minutes after an intravenous dose has been investigated. This clearance has been correlated with the presence of ^{14}C-labelled drug during that time.

2. Using labelled diethylcarbamazine, the uptake by organs and tissues in the rat has been examined at various intervals after an intravenous dose. No concentration above the average blood level was found constantly in any one organ or tissue.

The drug was not concentrated above the level in the incubating medium *in vitro* by rat red or white blood cells, or by adult worms of *Litomosoides carinii*, or microfilariae of *Dirofilaria immitis*.

3. None of the four metabolites of diethylcarbamazine—namely, diethylcarbamylpiperazine, methylpiperazine, piperazine, and the unidentified compound described in the previous paper—has any apparent effect upon the microfilariae of *Litomosoides carinii in vitro*.

4. The possible modes of action of this drug are discussed. The rapidity of clearance of microfilariae from the blood stream together with the other evidence described is consistent with the theory that the microfilariae are denatured or modified in some way so that they are treated as foreign particles.

It is a pleasure to thank Dr. F. Hawking for his continued interest and encouragement during the work in these two papers. I should also like to thank Miss W. A. F. Webber for supplying the infected cotton rats and Dr. A. D. Bangham for showing me the rapid sampling technique. I am most grateful to Mr. K. Hobbs for his patient and careful technical assistance.

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