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THE MAINTENANCE OF A FILARIAL INFECTION (*LITOMOSOIDES CARINII*) FOR CHEMOTHERAPEUTIC INVESTIGATIONS

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The purpose of this paper is to describe the maintenance in the laboratory of a filarial infection (*Litomosoides carinii*) which is suitable for chemotherapeutic and other types of investigations. The strain is kept in cotton rats (*Sigmodon hispidus*) and with one interruption it has now been maintained in our department for 2 years, during which time over 600 rats have been infected as shown by the presence of microfilariae in their peripheral blood.

HISTORY

Cotton rats began to be used in the United States for laboratory experiments about 1940; they were employed particularly in the study of viruses and rickettsiae. During more recent years these animals have been bred on a large scale in laboratories both in America and in Britain. Most of the rats used in the earlier work were wild ones which had been trapped in certain parts of Florida and Texas. Many of these wild rats were found to contain a filarial worm, *Litomosoides carinii*, which had previously been described in these and various other hosts by Travassos (1919), Mazza (1928), Chandler (1931), Ochoterena and Caballero (1932), Vogel and Gabaldon (1932), Chitwood (1933), and Vaz (1934); these are reviewed by Vaz. Culbertson and Rose (1944) showed that these spontaneous infections in wild rats were very convenient for experiments on the chemotherapy of filariasis, and much work was carried out by these authors (summarized by Culbertson, 1947) and by many other investigators in America holding O.S.R.D. contracts. The interest thus excited led to a determined search for the arthropod vector. Eventually, Williams and Brown (1945) showed that the worm developed in the tropical rat mite *Liponyssus bacoti* Hirst and transmission to clean rats was demonstrated by these workers (1946) and by Scott and Cross (1946). Descriptions of the morphology and life cycle of this mite have been given by Hirst (1913, 1914), Holdaway (1926), Dove and Shelmire (1931, 1932), and others. At the end of 1945 Dr. R. W. Williams, Dr. J. A. Scott, and other investigators kindly told one of the authors (F.H.) their latest results and demonstrated their methods for handling cotton rats. Dr. Williams and Dr. Scott kindly provided infected rats and mites which were brought back to Britain in February, 1946. Without their generous co-operation this work could not have been started. Further supplies of mites were kindly provided at a later stage by Dr. Cuckler, of Minneapolis, and by Prof. R. M. Gordon, of Liverpool. The objective of the work here described has been the development and practice of transmission on a large scale so as to obtain a good supply of infected cotton rats for chemotherapeutic investigations; during its progress, reports on various aspects of small-scale transmission have been published by Williams (1946), Scott (1946, 1947), Scott, Stembridge, and Sisley (1947), Hawking and Burroughs (1946), Bertram, Unsworth, and Gordon (1946), and Bertram (1946, 1947).

METHODS

The methods to be described were designed for transmitting *Litomosoides* to the maximum possible number of cotton rats with the minimum of labour. It is fairly easy to transmit this worm in the laboratory to a few animals at irregular intervals; but the maintenance of a constant supply of large numbers of infected animals is much more difficult, requiring constant care and attention to numerous small details. The chief difficulty is the prevention of foreign mites or insects entering the tanks which contain *Liponyssus* and rats, and destroying or overgrowing the *Liponyssus*. A second difficulty is encountered in the maintenance of proper conditions of humidity in the tanks, so that they are humid enough for the mites to

multiply but not so sodden with rat urine that the mites are drowned. The methods now employed have been subjected to repeated modification in the light of experience, and perhaps further improvements will still be found possible. Since success depends to such a large extent on the observance of small details, our present technique will be described rather minutely, after which the reasons for the various procedures will be discussed.

DESCRIPTION OF PRESENT TECHNIQUE

The process may be divided into five stages:

(1) The breeding of *Liponyssus bacoti* in pure culture.

(2) Infection of the mites with *Litomosoides* from infected rats.

(3) Infection of clean rats from the infected mites.

(4) Maintenance of the rats while the worms mature.

(5) Examination of the infected rats.

(1) *Breeding of the mites in pure culture*

The pure cultures are kept in the insectary described by Hunt and Davey (1947), in which the temperature is kept at approximately 24–26° C. and the relative humidity at 75 to 85 per cent. This room is used primarily for the maintenance of adult mosquitoes, but it is convenient to use it also for breeding the pure cultures of mites as the atmospheric conditions are ideal, the cultures occupy little space and, furthermore, they are isolated from contamination by foreign mites which may occur in the room used for the next two stages.

The colonies of mites are maintained in small glass jars (1-lb. jam jars) measuring approximately 8 by 13 cm. Before use the jars are washed. Clean sawdust about 2 cm. deep is put in the bottom of each, and on top of this a small piece of clean white filter paper is placed. The top of the jar is covered with finest bolting-silk or parachute nylon, the covering being tied round the neck with string. The jars and contents are sterilized in the autoclave (preferably the drying autoclave) and subsequently left 1–2 days in a 37° C. incubator to become thoroughly dry. The string fastening the silk covering is now removed from each jar and replaced by a rubber band. Mites (adults only) are removed from existing cultures by means of a fine camel's-hair brush (previously cleaned by dipping for a few minutes in ether) and floated off on to the surface of some clean water to remove any adherent matter. They are then transferred, again by brush, to a sheet of filter paper on which they are examined with a lens in order to make sure they are really *L. bacoti*, and that they have no smaller mites crawling on them, as is often the case. After examination they are transferred to the filter paper inside the jam jars. A thin film of dimethylphthalate smeared round the lip of a jam jar will prevent the escape of mites when the silk covering is removed. The effects of this repellent do not last more than one day, however. Each day one, or if possible two, day-old mice are put into each jam jar; or a day-old rat can be inserted every second day. To prevent contamination of the culture with unwanted species of mites which may be carried on the mice, each animal is placed in ether for about 10 seconds and then dried thoroughly in the air before being put into the jar. The mouse should be held gently with forceps throughout this operation. Mites feeding on a baby rat are shown in Fig. 1. Dead mice are removed daily with forceps, adherent mites being shaken from them back into the jar. Starting with ten mites in one jar it is possible in this way to breed about five thousand mites in a month, of which half may be expected to reach maturity.

A quicker but less safe method of "seeding" individual jars with mites is to remove a mouse from a jar which is already crowded with mites and to put it into the new jar. As the mouse will have a number of mites feeding on it at any given time, it is thus possible to carry over as many as a hundred mites in a single operation. However, mites should always be handpicked into one or two jars in order to maintain the stock culture, since otherwise there is considerable danger of contamination with foreign mites.

(2) *Infection of the mites with* Litomosoides *from infected rats*

This is carried out in an animal room set aside for the purpose. The temperature is kept between 27° and 32° C.; it is never allowed to fall below 24° C., as this will prevent *L. bacoti* breeding at the optimal rate. The relative humidity is maintained at about 50 per cent or 80 per cent, according to circumstances (see below), by means of a bucket of water standing over a small electric fire; the position of the bucket relative to the fire is adjusted when required according to the reading of a hygrometer on the wall. This simple arrangement is quite satisfactory for the present purposes. The mites (and rats) are kept in rectangular tanks, measuring 54×50 cm. by 26 cm. high, and made of 20-gauge galvanized iron sheeting (Fig. 2). The joints are brazed and riveted and the top edges of the sides are rolled; 4 cm. below the top edges, on the outside of each tank, is a horizontal ledge 1.7 cm. wide with an outer wall 1.3 cm. high, forming a channel which is normally filled with waste engine-oil. A rectangular wooden frame, of 3.6 cm. depth and 5.2 cm. wide, fits the top of each tank; on the under side there is a groove 1 cm. deep to fit over the edge of the tank; the recess is packed lightly with cotton-wool, which is soaked with liquid paraffin. Fine bolting-silk or parachute nylon is stretched over each frame and stuck down with petroleum jelly, and further secured by nailed laths. The joints of the wooden frame are packed with a plastic mixture of paraffin wax and petroleum jelly. Some metal tanks

FIG. 1.—Showing mites (the small dark objects) feeding on the posterior part of a baby rat.

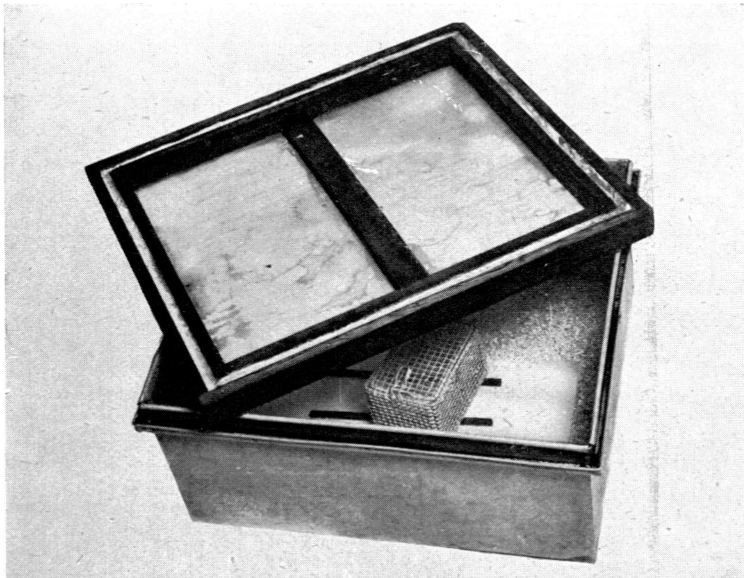

FIG. 2.—Metal tank with lid (inverted) used for maintaining the colonies of mites while in contact with infected rats. Note (1) the groove in the lid packed with white cotton-wool to seal the top of the tank, (2) the gutter filled with oil outside the tank, and (3) the rat cage standing on two pieces of wood, on blotting-paper, on sawdust.

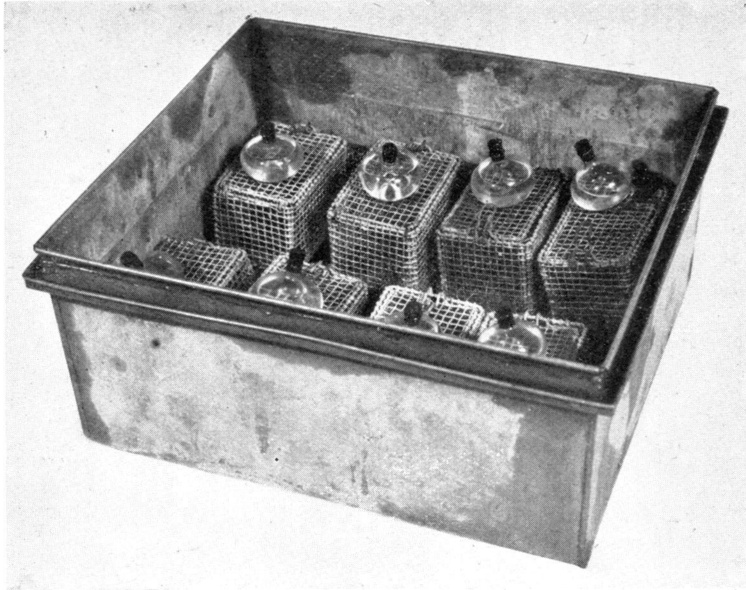

FIG. 3.—Metal tank containing eight clean rats in separate cages for exposure to infected mites; the glass sphere on top of each cage is a water bottle.

FIG. 4.—Room used for storage of rats while infection develops. The cages are stood in double rows on metal bars, with a tray of sawdust underneath to catch the droppings.

are also used of smaller dimensions, viz., 50 cm.× 40 cm. by 15 cm. high. The wooden lid of these is 10 cm. deep, so as to compensate for the shallowness of the tank. These second tanks do not have an external oil gutter. Consequently they must be stood on metal tables 125×60 cm. by 75 cm. high, which have an oil gutter 3 cm.×5.5 cm. deep round the margin, so as to prevent the escape of mites into the room. The tanks provided with an oil gutter are also stood on such isolated tables as a general rule, although they can also be stood on an ordinary table 75 cm. high. Wooden furniture is avoided as far as possible in this room, so as to avoid infestation with bed-bugs; if a wooden table is used, the legs are stood in small basins of oil or lysol.

Every effort is made to ensure that each unit (tank and lid) is proof against the entry of any small fauna —e.g., mites other than *L. bacoti*, lice, flies—which can prove dangerous to the life of *L. bacoti*. Nothing is put into the infection tanks which has not previously been sterilized or thoroughly cleaned. It is found useful to cover the hands and wrists with insect repellent (e.g., dimethylphthalate) before handling the tanks or their contents. Before use the tanks are steam sterilized. The lids are sprayed with xylene to kill any small fauna that may be present; they are placed on the tanks and allowed to dry. Sawdust sterilized in the drying autoclave is spread to a depth of about 5 cm. over the bottom of each tank. Several cotton rats infected with *L. carinii* and showing microfilariae in the peripheral blood are isolated in separate sterilized wire cages 15 cm. long × 10 × 10 cm. and sprayed with xylene from a scent-spray. (Great care is needed in order to avoid killing the rats with the xylene aerosol.) The cages are supported on clean metal rods resting on two opposite sides of a shallow tray containing dilute lysol, and the rats are left there until all trace of xylene fumes has disappeared. The cages, with enclosed rats, are then placed in the tanks containing the sawdust. Four thicknesses of dry sterilized blotting-paper are placed under each cage to catch excreta. The cage is raised about half a centimetre above the blotting-paper by being laid across two small sticks, so that accumulated faeces do not soil the bottom wires of the cage. The blotting-paper is changed every two days. Usually two infected rats are allotted to each tank, but more may become desirable if mites are very numerous. Mites are added to the tank by pouring into it the contents of one or more of the jars containing mite-colonies described in the previous section.

During their sojourn in the tanks the rats are kept on a minimum diet. Two to four grammes of cubed processed food, previously baked to sterilize the surface, and ten cubic centimetres of water per rat daily are sufficient. Particles of waste food must not be allowed to accumulate, and excess of urine must be avoided. Since the rats are serving to encourage breeding of the mites, as well as to infect them, they are left in the tanks for seven days, or until the mites are "swarming" (not only near the rats, but also over the whole of the sawdust), whichever time is the greater. (The mites show great activity if the observer breathes on to the sawdust.) The rats are then removed from the tanks and are "demited" passively by being left for a two-day period over the tray of lysol described above.

(3) *Infection of clean rats from infected mites*

The same tanks are used as in the previous section. Within three days of taking out the infected rats, two or three cages measuring 20×28 cm. by 10 cm. high are placed in the tank. Each cage contains five young female rats which have been freed from foreign mites by means of xylene as described above. Alternatively, eight of the small cages containing single rats may be inserted (Fig. 3). The blotting-paper is changed every day except Sunday to prevent the accumulation of urine and faeces. The soiled blotting-paper and the faeces lying on it usually contain many mites; in order to allow these to fall back on to the cage, the paper and its adherent faeces are stored for one or two days on top of the cages inside the tank before they are thrown away. The positions of the cages inside the tanks are transposed every day so as to compensate for any maldistribution of mites. The humidity of the room is adjusted according to the number of rats present. If few are in the tanks, the relative humidity is raised to 80 per cent; if many are present in the tanks, the humidity is allowed to fall to 50 per cent in order to encourage the drying of the blotting-paper and sawdust. A few days after the clean rats have been inserted, about 30–40 mites may be removed from the tank and dissected to look for worms. This confirms that the mites will become infective, otherwise the further procedure must be modified accordingly.

After fourteen days the clean rats are removed and demited as was described above. If the empty tanks seem well stocked with mites and free from contamination by foreign fauna, infected rats are inserted in order to infect the mites again, thus re-starting the whole cycle. Otherwise the contents of the tank are placed in an incinerator and the tank is cleaned and sterilized, after which it will be available for use as at the beginning.

(4) *Maintenance of the rats while the worms mature*

After the rats have been removed from the infection tanks and demited, they must be stored for about fifty days while the worms mature. During this waiting period, female rats can be kept in cages of five; males must be kept in separate small cages (15×10× 10 cm.). All cages have wire bottoms. They are arranged across the longitudinal bars of a big metal rack (Fig. 4). A few centimetres below each row of cages there are long metal trays to catch the excreta. The trays contain sawdust and are changed every two days. The rats are provided with an excess of the Institute cubed stock diet for rats and mice, supplemented by green cabbage three times weekly. Water is provided in excess in licking-bottles. The temperature of the room is maintained at approximately 25° C.

(5) *Examination of the infected rats*

Cotton rats are more difficult to handle than ordinary laboratory rats, since they are easily frightened and try to bite when gripped ; also the skin on the tail is too fragile for them to be lifted up by this means. We have found that the most satisfactory technique is to approach the rat suddenly from behind and grasp it firmly by the scruff of the neck. A worker with sufficient confidence in himself can do this with the bare hand, without risk of injury ; others will require stout leather gloves to give them a feeling of safety. An alternative technique is to take hold of the body by the hand protected by a leather glove so that the head projects between the thumb and forefinger. The rat twists round to bite the glove near the base of the thumb, but with a suitable glove there is so much slack leather at this point that the teeth do no harm. Meanwhile, blood can be easily taken from the tail or injections can be made into the posterior half of the animal. If there is difficulty in extracting a rat from a small cage, the rat is emptied out into a small dustbin more than 80 cm. deep—i.e., too deep for the rat to jump out. In this it can usually be grasped easily.

About eight weeks after removal from the infection tanks a drop of blood is taken from the tail of each rat and smeared on a slide to make a thick film, about 2 sq. cm. in area. These films can be stained by any of the usual techniques for microfilariae. We have found it rapid and convenient to dry them, to dehaemoglobinize in 1.5 per cent acetic acid, to dry again, and to stain with Leishman's stain.

After examination each rat is kept in a separate small cage. Rats which fail to show microfilariae three months after removal from the infection tanks are considered not to be infected, and they can be exposed to infection once again. We have no reason to believe that such rats are protected by a natural immunity, but the matter requires further investigation. If the number of infected mites in the tanks is high, there should be few failures to infect rats.

Comments on the Technique for Obtaining Rats Infected with *Litomosoides*

The previous section has described, somewhat dogmatically, the technique at present in use. Various features of this technique may now be discussed in more detail, to show the reasons for adopting them and our experience with alternative procedures.

(1) *Breeding of mites in pure culture*

The technique (described above) for breeding mites in jam jars has been in use for a year. It was started at a time when the mite population at our disposal was dangerously low, and the first jars contained soil and hay in imitation of the rat infection boxes then in use. Our whole present population is derived from three jars of one dozen mites each. We have not once had a jar contaminated with foreign mites. It is considered that this technique is more convenient and safer from contamination than the Single Unit technique described by Bertram, Unsworth, and Gordon (1946) or than methods requiring the isolation of mites after every feed.

Some observations on the habits of these mites are made possible by this technique. If the mites are well fed, with an excess of hosts (mice) present, they will not be seen above the sawdust except when actually feeding. Examination of the sawdust with a lens will reveal numerous stationary adults and highly active unfed protonymphs making their way upwards. Where the mites have insufficient food, however, they run rapidly over the inside of the jar, and will congregate on the inner side of the silk covering if there are mammals in the room. As soon as a mouse is put into the jar, further large numbers of mites will emerge from the sawdust and run rapidly about in the vicinity of the mouse. They appear to be under the influence of a simple chemotaxis which causes them to move directly towards the source of stimulation, until within one or two centimetres of it, when they will skirt about for some minutes before arriving at the source itself. Once on the mouse, young protonymphs will begin to feed almost at once. The older mites will run over the skin for as long as half an hour, before taking up a position to feed, usually at an orifice or in a fold of the skin. Behind the ears, in the groin, in the fold of skin between the shoulders, and around the mouth and nostrils are the favourite feeding places. The mites appear to stop running when they touch a feeding mite, and not infrequently start feeding themselves. In this way little clusters of mites soon form (Fig. 1). The older mites do not seem to interrupt their feeding as frequently as the protonymphs do.

(2) *Infection of the mites from infected rats*

As was said above, the maintenance of healthy colonies of mites during this and the next operation depends chiefly on the avoidance of contamination by other small fauna and the maintenance of appropriate humidity in the bedding. The tanks used were at first uncovered, but contamination with foreign mites persistently developed in spite of all precautions to prevent the accumulation of waste food and faeces. Even with the covers, most tanks become contaminated within six weeks of setting up. When contamination occurs, the

contents of such tanks should be sterilized as soon as the cycle of operations can be profitably terminated, since the population of *Liponyssus* usually diminishes rapidly, and the tank acts as a source of contamination for other tanks.

The commonest contaminants are mites of genera other than *Liponyssus*. When silk covers for the tanks were not used, the forage mite, *Tyrophagus castellanii* Hirst (and probably other similar species), was most frequently found, swarming over the fragments of food dropped by the rats, and on the licking-bottles. These mites had no appreciable effect on the *L. bacoti*. Less frequently a large yellow mite (adults 1.0 to 1.5 mm. long), *Macrocheles carinatus* Koch, would appear, and its appearance always heralded a decrease in the numbers of *L. bacoti*, so that we were led to the conclusion that this mite would eat either *L. bacoti* or its eggs. More recently a smaller yellow mite (adults 0.5 to 1.0 mm. long), *Hypoaspis freemani*, has been a frequent visitor, with similar disastrous consequences for *L. bacoti*. We have had little trouble from the Insecta. Lice have been seen once or twice, presumably feeding on the rats, but never in great numbers and never to the detriment of *L. bacoti*. A Dipteran, *Coprophila vagans* Hal. (a small black fly superficially resembling *Drosophila*), troubled us before the silk covers were introduced. It bred among the rat faeces and probably carried other contaminants from one tank to another.

The blotting paper underneath the cages was introduced in order to catch the urine, faeces, and waste food from the rats and to reduce soiling of the bedding. Pieces of cardboard were first employed and were moderately satisfactory; but they were difficult to clean and too expensive to renew every two days. Metal plates were tried but they collected pools of urine, in which the mites drowned, so they were quite unsuitable. Initially the waste material from the cages matted together in the meshes of the wire floor and formed an impervious sheet; this was obviated by inserting a small wooden bar, about 0.5 cm. thick, between the blotting paper and each end of the cage. The blotting paper should not project more than about 1 cm. round each cage, lest it hinder passage of the mites between the cage and the underlying sawdust. Young rats tend to gnaw the paper through the floor of the cage, reducing the upper surface to a powdery mass; this does little harm, apart from the mechanical inconvenience. The supply of drinking water to the rats has to be adjusted so as to prevent the blotting paper becoming soaked with excess of urine—otherwise conditions become unfavourable for the mites. The amount of urine which can be tolerated depends on the humidity of the atmosphere, on the number and size of rats in the cage, and on the frequency of changing the paper.

The bedding used in these cages originally consisted of a layer of dry sterile earth covered with sterile hay or straw as described by Williams (1946). Sawdust was found to give equally satisfactory results, with the additional advantages of cleanliness and the provision of a good background against which mites could be easily seen. The rats used to infect the mites should contain numerous microfilariae: the blood of our donors usually contains more than 50 microfilariae per cu.mm. If the mites are too numerous they may cause death of the rats by exsanguination; the danger-limit can be recognized only by experience. When it is approached, the donor rats can be changed, more donor rats can be added, or as a last resort the number of mites may be reduced by judicious spraying with xylene.

The percentage of mites which develop filarial infection during our procedure is small. Sample dissections have been done at various periods after exposing rats to the mites for 7–14 days. In 716 mites dissected within 6 days of the last day of exposure, 4.3 per cent were infected; of 419 mites dissected between the 7th and 13th days after exposure 1.4 per cent were infected, while of 240 mites dissected 14 or more days after exposure, none were found infected. For various reasons, these percentages are probably lower than the actual rates among the mites.

The length of the worms found in these mites varied from 60 μ to 800 μ, according to the period which had elapsed since the worm was ingested by the mite. This infection rate of the mites is much lower than has been reported by some observers—e.g., Bertram (1947) speaks of infection rates of 38 per cent. In our colonies the mites which have fed on the infected rats are constantly being diluted by young mites which have not so fed. All the same, there are sufficient infected mites in the colonies to transmit the parasite readily, and even in tanks where 50 or 100 mites were dissected without finding worms 50–100 per cent of the exposed rats became infected.

(3) *Infection of clean rats from infected mites*

The general problems of this stage are the same as those of the preceding stage. Greater care is required to prevent the blotting paper and bedding getting too wet. The caging of the cotton rats during this and the subsequent stage depends on their age and sex. The rats are most conveniently kept in groups of five, since each rat has more

freedom and the whole group occupies less space than five separate small cages. Unfortunately, confined groups of adult male rats or of mixed sexes are usually decimated by savage fighting ; so that when pubescent or mature males are used they must be kept in separate small cages (Fig. 3). Restriction of use to female rats is convenient for handling, but it is rather wasteful of the breeding facilities.

(4) *Maintenance of rats while the worms mature*

The chief difficulties encountered during this phase arise from the number of rats involved and the problem of intercurrent mortality. If two dozen rats are removed from the infection tanks weekly and each must be stored eight weeks, accommodation is required for about two hundred rats, many of them in single small cages. The arrangement described (Fig. 4) is the most compact, most convenient, and most economical of attendant labour that we have yet been able to devise. At various periods there have been many deaths among our rats, sometimes averaging two or three per day, so that a quarter of our rats were being lost in this way before the infection matured. The causes of these deaths could not be satisfactorily determined. The distribution on the animal racks was not suggestive of contagious spread. Post-mortem examination of dead rats showed reddened intestines in some, thrombosis in the left auricular appendage of the heart in others, while others showed a clear effusion in the pleural cavities ; intranasal instillation of this effusion into two other rats caused no obvious symptoms. Histological sections from a few of the rats suggested an infection with *S. typhi-murium*. Feeding experiments showed that growth was improved by adding green food to the diet, but otherwise the stock cubed diet for rats of the Institute seemed adequate. It was concluded that such sporadic deaths were best combated by attention to the general hygiene and diet.

(5) *Checking the rats for the presence of microfilariae in the peripheral blood*

Microfilariae do not appear in the tail blood of infected rats until 42 days after their removal from the infection tanks. (We habitually examine the rats for the first time after this period.) This means a possible maximum of 56 days from the first exposure to infection, and agrees with the conclusions of other workers that 51–54 days is the time required for microfilariae to appear. With light infections the appearance of microfilariae may be delayed until the 55th day after removal from the infection tanks. This may mean that the rats were not bitten by infected mites until their last day in the tanks, or that the number of microfilariae in the blood was too low to show in the first blood samples.

With most of our rats counts of microfilariae taken between the 40th and 60th days show a fairly consistent density of 10 to 15 per cubic millimetre of tail blood (about 2 per microscope field (× 60) of an average thick smear) ; thereafter the count increases to between 100 and 300 per cubic millimetre at the 100th day, and it remains nearly constant after that (so long as the adult worms are healthy).

ADULT *L. Carinii* IN THE HOST

We have never found adult worms in any part of the host other than the pleural cavity (and occasionally the mediastinum). However, we have occasionally failed to find worms in their normal location, although the blood of the host was positive for microfilariae, and so the possibility of successful development of the worms outside the thorax cannot be excluded.

Healthy worms in a healthy host tend to spread thinly over the wall of the pleural cavity, adhering by capillarity to the moist surface. They are seen to wriggle continuously in the dissected host ; perhaps they are less active if undisturbed. If there is fluid in the cavity, the worms sink under the influence of gravity and become matted together. They also become matted if subjected to unfavourable conditions, e.g., if the host is treated with antifilarial drugs. When killed by drugs the mat of worms is soon covered with a fibrous deposit. In rats which have been infected for more than three months we have occasionally found spontaneous cures. Here, as in the drugged rats, we have found the worms matted together and covered with fibrin. In order to avoid mistaking such spontaneous cures for the results of drug action therapeutic tests are best performed with newly infected rats.

The worms are best examined in their living state. After removal from the pleural cavity the worms should be immersed in warm Ringer's solution and put in a 37° C. incubator for half an hour. At the end of this time normal worms show active motility, particularly in a bright light. They are nearly transparent, and most of their internal structure can be made out with care. We have noticed that after treatment with drugs of varying degrees of potency the behaviour and appearance of the worms are modified in a fairly constant fashion. The weakest effective drugs (or doses)

induce sluggishness, while stronger ones result in reduced transparency of the worms' tissues. When the worms are actually dying as a result of therapy they become covered with a thin layer of phagocytes, and later with fibrin, and they acquire a brown coloration. Even at this stage they may retain some motility. These changes are best seen a week after the course of injections has terminated, and they may be absent in rats killed earlier than this.

A moderate number of worms in the pleural cavity does not induce any gross pathological changes. If the number is excessive, however, the surface of the lung becomes inflamed and purulent. This may be due, to penetration of the tissue by microfilariae.

Spontaneous death of the worms has never been found to occur in less than three months after removal of the rats from the infection tanks. When it does occur, the dead worms are usually found in a dense capsule of fibrin, and they exhibit little or none of their normal structure.

Transmission of Litomosoides *to animals other than cotton rats*

In the early descriptions of the worm now called *L. carinii* it was reported in various other small rodents, viz., *Sciurus*, and *Hesperomys* (Travassos, 1919), *Holochilus vulpinus* (Mazza, 1928), and *Nectomys squamipes* (Vaz, 1934). Accordingly, an investigation was made of the possibility of using other small rodents as experimental hosts for *Litomosoides* in place of the cotton rat. Animals were exposed to infection by the technique described above and the results are summarized in Table I. A preliminary note on these findings was published by Hawking and Burroughs (1946).

TABLE I

SUMMARY OF ATTEMPTS TO TRANSMIT *Litomosoides* TO ANIMALS OTHER THAN COTTON RATS

Species	Number of animals		
	Exposed	With worms in pleura	With Mf. in blood or pleura
Piebald rat ..	23	5	3
White mouse ..	70	11	2
Guinea-pig ..	2	0	0
Hamster .. .. (*Cricetus auratus*)	3	3	1
Orkney vole .. (*Microtus orcadensis*)	5	1	0

The piebald rats and mice which did become infected were consecutive ones out of a series spread over many months; this suggests that if the others had been exposed to mites as heavily infected, they also would have developed worms. Some of the animals contained up to thirty worms, but most of them contained less than ten, both of which numbers are low for cotton rats. In piebald rats the microfilaria count in the peripheral blood rose to 1,000–5,000 per c.cm., and in two of the rats they persisted for more than two and three months respectively; the rats were then killed. In all the animals of Table I the infections were much lighter than those usually found in cotton rats and there seemed to be a tendency towards spontaneous cure. It was concluded that although *L. carinii* will obviously develop in quite a variety of rodents (as shown by the literature and our own results) none of the species studied formed an efficient substitute for the cotton rat, which in fact remains the only satisfactory animal for chemotherapeutic experiments on this worm.

CHEMOTHERAPEUTIC TESTS ON INFECTED RATS

In designing a chemotherapeutic test consideration must be given to (1) allowing the drug sufficient opportunity to show its antifilarial activity (if any); (2) avoiding undue consumption of laboratory time, labour, and material. Culbertson and Rose (1944b) have shown that the effect of treatment of *L. carinii* infections with antimonial compounds must be judged by examination of the adult worms, since the microfilariae of the peripheral blood are not affected by doses which kill the adults. On the other hand, Hewitt and his colleagues (1947) have recently reported that piperazine derivatives act mainly on the microfilariae and only to a much smaller extent on the adult worms. Accordingly, examination of both adults and microfilariae is necessary in judging the antifilarial action of an unknown compound. Our preliminary experiments with neostam (Table II) suggested that treatment given on six successive days was more effective than treatment on three days only; but treatment on twelve or eighteen days (which used much more time, labour, and drug) was not obviously more effective than treatment on six days. In view of these results and of reports by American workers, it was decided to utilize a dosage schedule of six daily doses. The procedure adopted is as follows:

When a compound is to be tested, a rough estimate is first made of its chronic toxicity for mice when given by intraperitoneal injection once daily for four successive days. Two mice are used per dose and each dose is approximately twice the

TABLE II

SPECIMEN RESULTS OBTAINED IN TESTING COMPOUNDS FOR FILARICIDAL ACTIVITY

Compound	Max. tolerated daily dose for mice (chronic toxicity) mg. per kg.	Treatment of rats				Condition of adult worms*
		Dose mg. per kg.	No. of doses	Interval (days)	No. of rats	
Neostam .. (stibamine glucoside)	250	40	6	1	2	Majority alive; a few dead
		40	18	1	2	All alive
		80	6	1	1	All alive
		80	12	1	2	All alive
		130	3	1	2	All alive
		130	3	2	2	All alive in one case; all dead in the other
		130	6	1	2	All dead in one; majority dead in the other
		130	12	1	2	All dead in one; majority dead in the other
		160	3	1	2	All dead
		250	6	1	1	All dead
		260	2	3	2	All dead in one case; all active in the other
		260	1	—	1	All dead
Pentostam .. (sodium stibogluconate)	2,500	200	6	1	1	Females injured; males unaffected
		500	6	1	1	All dead except a few of the males
		1,300	6	1	1	All dead
Anthiomaline ..	100	45	6	1	2	Males unaffected; females dead
Tryparsamide ..	2,500	250	6	1	2	Majority alive; a few females dead
		500	6	1	2	Males alive; females dead or dying
Neoarsphenamine	250	100	6	1	2	All dead
p-Methylsulphonyl benzamidine hydrochloride (V 187)	250	20–50	6	1	3	All alive

* Microfilariae were unaffected in all experiments

one below it. If the highest dose which has not killed any of the mice is x mg. per kg., the dose used for the therapeutic trial in cotton rats is $0.4x$ mg. per kg., given by intraperitoneal injection for six successive days; this dosage seldom proves toxic for the cotton rats. If the compound is found to be active, its toxicity can be investigated more closely. Infected rats are taken and a rough estimation is made of the number of microfilariae in the blood by counting the larvae in several typical microscope fields of a thick blood film. The rats are then treated by intraperitoneal injection given once daily on six successive days. Cotton rats infected with *L. carinii* do not tolerate the intraperitoneal injection of drug quite so well as healthy rats do. Also cotton rats are more susceptible than other laboratory animals to sepsis and bacterial infections during this procedure. All drugs should be dissolved in sterile fluid, and kept in sterile containers. Syringes and needles should be sterilized before use, and the site of the injection should be well cleaned with cotton-wool soaked in ether or in dilute alcoholic iodine solution before performing the injection.

Six days after the last dose the blood is examined again for microfilariae, the rat is killed, its thorax is opened, and the worms found in the pleural cavity are removed and examined. If their

motility is not obvious they are suspended in Ringer's solution and warmed to 37° C., which often stimulates them to motility. Often a mass of worms is found and it may be necessary to examine them under a microscope in order to estimate the proportion of living and dead ones. After effective filaricidal treatment, e.g., large doses of antimonials, the worms are usually found embedded in fibrin, forming small compact white masses. The microfilariae are much more resistant to treatment (by compounds other than piperazines) than the adult worms are, and when the adults have been killed by antimonials many active microfilariae can be found in the pleural fluid of the rat; living microfilariae also persist in the peripheral blood, where we have found them in gradually diminishing numbers for more than three months (as described by Culbertson and Rose, 1944b).

Examples of some of the therapeutic tests are reproduced in Table II. As reported by other observers, adult worms can be killed by suitable doses of antimonials (e.g., neostam, pentostam, or anthiomaline) and of arsenicals (e.g., tryparsamide). The males usually seem more resistant than the females. The microfilariae are unaffected by these types of compound. Most of the substances examined to date have unfortunately been inactive, e.g., V 187.

Discussion

In 1940 one of the authors (Hawking) described unsuccessful therapeutic investigations on patients with filariasis in East Africa, and pointed out that, although a chemotherapeutic remedy for filariasis would eventually be found, the testing of compounds directly on human beings was unlikely to produce the desired solution on account of the many difficulties and limitations inherent in such work; in order to be able to seek such a remedy with good prospects of success it was necessary first to obtain "an animal carrying a filarial infestation suitable for laboratory experiment; thus the animal must be small and cheap . . . and the problem of transmitting the infestation, presumably by insect vector, must also be solved." The infestation of cotton rats with *Litomosoides*, described above, seems to fulfil the conditions then laid down, and the prophecy then made that when this postulated experimental animal was available "the discovery of a potent filaricidal substance would be only a matter of time" is apparently in the process of fulfilment by the piperazine derivatives reported by Hewitt and his colleagues (1947).

The filarial infections hitherto available in the laboratory have consisted of parasites of dogs, lizards, and frogs. The filariae of dogs include *Dirofilaria inmitis* and *D. repens*; but dogs are too large and costly to keep in any quantity, naturally infected animals cannot easily be obtained in Britain (although they are available in the United States and Mediterranean areas), and transmission in the laboratory takes about ten months. Although dogs offer advantages for the study of the periodicity of microfilariae and for certain other studies on these stages of the worm, they are too big and expensive for the screening of new compounds. Filarial infections have been studied in lizards by Menon, Ramamurti, and Rao (1944), in frogs by Witenburg and Gerichter (1944), and in birds by many observers; but these infections are not easily available in Britain, transmission in the laboratory is difficult or impossible, and the metabolism of cold-blooded animals may be different from that of warm-blooded ones, so that experimentation in these hosts would be an unreliable guide to what might happen in man. Spontaneous infections of cotton rats with *Litomosoides* have already made possible investigations of the antifilarial action of antimonials (Culbertson, 1947, Culbertson and Pearce, 1946, and Culbertson and Rose, 1944a and b), of mercury and other compounds (Lawton *et al.*, 1945a and b), of the cyanine dyes (Welch *et al.*, 1947), and of the promising piperazine derivatives (Hewitt *et al.*, 1947; Santiago-Stephenson, Oliver-Gonzalez, and Hewitt, 1947). It is hoped that the technique described for transmitting the infection in the laboratory may render possible similar investigations in Britain and other countries where naturally infected rats are not available. Even where naturally infected rats are available, laboratory infections will probably prove preferable, being cheaper, more standardized, and more readily available in large numbers.

Summary

Large-scale methods are described for the breeding of *Liponyssus bacoti* and the transmission of *Litomosoides carinii* to cotton rats. During 1947 over 400 rats were infected by these methods in the authors' laboratory. These rats are used for screening compounds for antifilarial activity and the procedure employed is outlined.

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SUPPLEMENTARY NOTE

Since this text was written the following modification of technique has been introduced: Blocks of plaster-of-Paris 2.0–2.5 cm. thick are used to absorb urine and collect faeces in the infecting tanks; they take the place of the blotting-paper mentioned on p. 289. Each block is of such a size as to accommodate four small cages or one large cage with 1–2 cm. overlap. Faeces are periodically scraped off each block into a perforated zinc tray which hangs from the side of the tank; mites, which are usually plentiful in the faeces, can then return to the sawdust. Between periods of service in the tanks, which may be of up to two months' duration, the plaster blocks are steeped in water and then dried, when they are ready for use.

An important paper (Williams, R. W., 1948, *J. Parasit.*, **34**, 24) entitled "Studies on the life cycle of *Litomosoides carinii*" appeared early this year, too late to be discussed in the text.