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INACTIVATION OF ASCARIS LUMBRICOIDES EGGS BY HEAT, RADIATION, AND THERMORADIATION

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ABSTRACT

It is desirable to eliminate the public health hazards associated with land application of municipal sewage sludge as a fertilizer or soil conditioner. This report describes experimentation to determine the effects of heat, radiation, and thermoradiation on the suppression of embryonation of Ascaris lumbricoides ova, a parasite commonly found in sewage sludge. Heat effects were observed at a minimum temperature of 51°C and radiation effects at doses in excess of 15 krads of ionizing gamma radiation. Thermoradiation at 47°C suppressed embryonation at less than half the total dose required by radiation alone.

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INTRODUCTION

The presence and control of parasite eggs in sewage sludge presents a problem when the sludge is used for agricultural purposes. The fertilized eggs of Ascaris suum, for example, may remain viable in moist soil for four to five years (1). Liebmann and his students (2) have found that cattle brought into pastures irrigated by insufficiently cleaned sewage have unusually heavy infestations of this parasite. Chandler (3) reports that a German researcher seeded a plot with Ascaris eggs; two persons ate unwashed strawberries from the plot each year for six years and each year acquired a few Ascaris.

Of the parasite eggs found in sewage, those of Ascaris species are most common, followed by various Trichocephalus, whipworm, species (4). Species from the Necator and Ancylosterna families are more rarely found. Geographic factors influence the families represented in sewage and sludge samples but the ubiquity of Ascaris places quantities of their eggs in all sewage or sludge tested (4, 5).

Cysts of Endamoeba Histolytica are also found in great quantities in sewage and sludge. However, the concern for survival of these cysts in sludge used for agricultural purposes is minimal. Wang and Dunlop (5) report that field grown crops contaminated with cysts of Endamoeba Histolytica are safe for raw consumption two weeks after contamination.

Ascaris infections are among the longest known of human parasites. Its life cycle has been explored and well defined. This large nematode in the

adult form is found in the small intestine. The females can reach a length of 20 to 35 cm and males 15 to 30 cm. The egg production of the female ascarid ranges from 200,000 to 250,000 eggs/day and is excreted in the feces of the host. These unsegmented eggs range in size from 60 to 70 μm by 40 to 50 μm and are covered by a thick albuminous coat. For the eggs to develop they require an environment with the temperature lower than the host body, a trace of moisture, and a supply of oxygen. If the eggs do find themselves under favorable conditions, active embryos develop in a week and the eggs reach an infective stage in 17 to 34 days. Should the eggs then be ingested, the larvae hatch in the small intestine, where they penetrate the mucous membrane and travel throughout the body to the liver, heart, and/or lungs. Eventually the worm settles in the small intestine of its host.

A combined heat and irradiation treatment has been proposed for sewage sludge intended for land usage (6). In addition to inactivation of pathogenic microorganisms, for the reasons outlined above, it is desirable to assure that a sewage treatment also inactivates parasite eggs. The ubiquity of Ascaris, the presence of large quantities of their eggs in sewage, their resistance to chlorination (7), the tendency of eggs to concentrate in sludge (4), and their reported resistance to heat (3) make Ascaris a logical choice for parasite egg-sludge inactivation studies.

MATERIALS AND METHODS

Preparation of Non-Embryonated Eggs

Ova were obtained from pregnant female *Ascaris lumbricoides* worms which measured 27-36 cm in length (Carolina Biological Supply Co., Burlington, NC). About 5 cm of the posterior end of the uterus was removed from each worm and placed in 1N NaOH. After all uteri had been collected, they were mashed into small pieces, about 1-3 mm in length, with a glass rod and stirred for 30 minutes with a magnetic stirrer. The mixture was poured through a 48 mesh (295 μm) screen into a flask to remove uteri fragments. After settling for 40 minutes, most of the NaOH was decanted. The ova were washed twice more in the same manner, first with fresh NaOH and then with deionized water, after which 0.1N H_2SO_4 was added to the suspension and the ova stored at 4°C.

Preparation of Samples

The concentration of ova in the stock suspension was determined by counting the number of ova in 0.1 ml using a McMasters Chamber and a Leitz binocular microscope at about 125X. Samples in which the ova were to be exposed to heat, irradiation, or thermoradiation in a deionized water suspension were prepared as follows. One ml of the stock suspension was placed in a 16 x 150 mm screw cap test tube. The suspension settled for 30 minutes, 0.5 ml of the 0.1N H_2SO_4 was removed, and 4.5 ml of deionized water was added to the tube. For those experiments in which the ova were exposed in a sludge suspension, 3.5 ml of water and 1.0 ml of sludge supernatant (1% solids) were added to the tube.

Irradiation

Irradiation was accomplished in the Sandia Gamma Irradiation Facility (GIF) using a 92 kilocurie Cobalt-60 source. Most of the experiments were conducted using dose rates of about 18 to 36 krads per minute. Thermoluminescent dosimetry (TLD-400's) was used to measure absorbed dose in each experiment. The ova were irradiated at ambient temperature of 14.5 to 22°C and in concentrations ranging from 3×10^3 to 10^4 per ml.

Thermoradiation

Thermoradiation experiments were also conducted in the GIF. In this case, the sample tubes were placed in a temperature controlled water bath during the time of exposure to radiation. The water temperature was controlled to ± 0.1°C. Thermoluminescent dosimetry was also used to measure absorbed dose in each of these experiments. The ova were irradiated at temperatures ranging from 47°C to 60°C and in concentrations ranging from 3×10^3 to 10^4 per ml.

Heat

Sample tubes exposed to heat were placed in a temperature controlled water bath with a shaker fixture to provide mild agitation. Water temperature was controlled to ± 0.1°C. At the end of the exposure period, the tube was removed from the shaker and immediately placed in ice water for 30 seconds to lower the temperature rapidly to ambient. At the same time, the upper portion of the tube, above the water line of the suspension, was flamed to inactivate any ova which may have been attached to the inner surface above the water line and not exposed to the same degree of heat as those ova in the suspension.

Embryonation

After exposure to an incubation environment, the water was decanted and 100 ml H_2O_{dd} was again added to the tubes as the embryonation media. The ova were incubated for 21 days at 30°C on a roller drum which revolved at a speed of 1 revolution in 5 minutes and 15 seconds.

Counting Procedures

Following embryonation, the tubes were shaken vigorously to evenly disperse the ova throughout the suspension. Samples were extracted from the tube and placed in a Hollister Chamber for counting under the Leitz microscope. 3 ml counts were made from each sample with each count representing 0.1 ml of the suspension. Counts included embryonated and non-embryonated ova; non-fertilized ova were disregarded. Ova were considered embryonated only if embryonation had reached the larval stage and the larva was clearly visible within the ovum. Similar counts were made on control (non-exposed) tubes and the data normalized to establish the control embryonation at 100%.

General Laboratory Equipment

All ova cleaning procedures, sample preparation, and slide preparation were conducted in a laminar flow bio-safety cabinet with a HEPA-filtered air exhaust. Worms were dissected under water and uteri were removed in a fume hood to decrease operator exposure to ascaryl. Test tubes containing samples were always wiped with an ovacide solution of 50% xylene and 50% ethanol (9) and the cap end sealed with parafilm when they were removed from the bio-safety cabinet.

Here

Fertilized Ascaris eggs were distributed in [illegible] environments ranging from -7°C to 72°C for various periods of time. [illegible] were observed from the -7°C temperature after various [illegible] a water bath was increased to [illegible] was unaffected by 15 minutes exposure [illegible] 15 minutes exposure, but 5% of [illegible] time for the development [illegible] <1% at 45 minutes. In contrast, eggs [illegible] (control = 100%) after 5 minutes exposure [illegible] 75% and 0% were noted in hours.

Larvae within [illegible] of fertilization [illegible] at various temperatures [illegible]

$t_{.5}$ = >. hrs.

$t_{.1}$ = .. min.

$t_{.2}$ = . min.

$t_{.5}$ = .. min.

A similar experiment initiated at 5:15, on Wed., Nov. [illegible] ment subjected for 1 hr. 15 min. water circulating, then [illegible] 15 minutes.

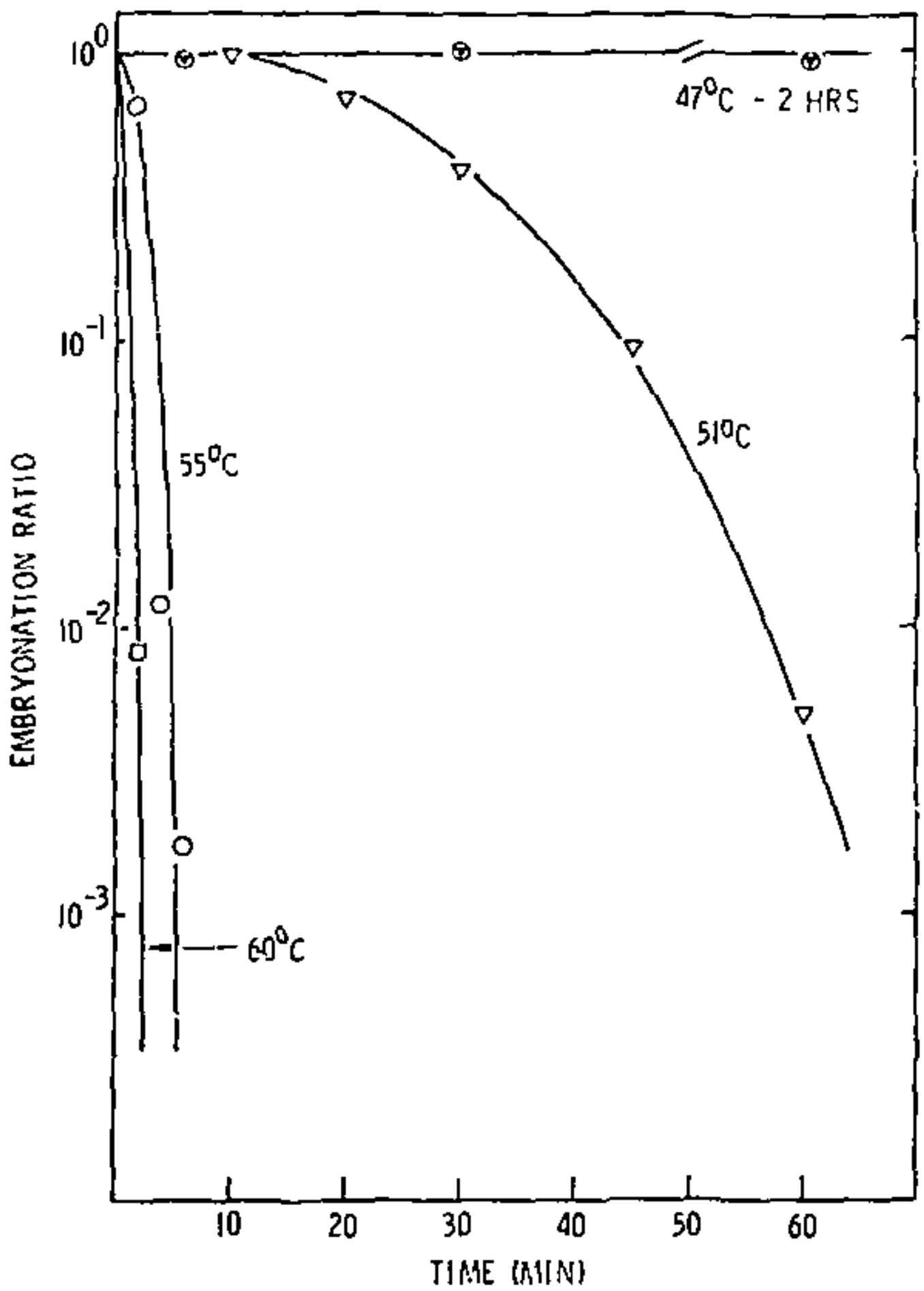

Figure 1. Inactivation of Ascaris ova by heat. Observations are indicated by ⊕- 47°C, ▽- 51°C, O- 55°C, and □- 60°C.

Irradiation

Most of the Ascaris ova were in a H_2O suspension during irradiation. The irradiation curve in Figure 2 is typical of the irradiation effect on embryonation inhibition. A slight shoulder was observed up to a dose of ~15 krads, after which the irradiation effect became logarithmically evident as shown by the following embryonation data:

1st log decrease - 46 krads

2nd log decrease - 21 krads

3rd log decrease - 14 krads

No embryonation was noted at a total dose of 95 krads in a sample of 2.5×10^3 ova.

In another set of irradiation experiments using [illegible] batch of worms, the shoulder effect was more pronounced, increasing the radiation dose required for each log decrease in embryonation. About 90 krads required for the first log reduction, with the inactivation proceeding logarithmically after that point, with the second log requiring 25 krads and the third log 17 krad. Dose rate effects appeared to be negligible.

Other experiments were conducted to determine whether [illegible] suspension offered any protection to the ova during irradiation. Virtually no difference in embryonation was detected. In fact, the inactivation curves in Figure 3 are nearly identical.

Thermoradiation

Thermoradiation (simultaneous application of heat and irradiation) experiments were conducted at both 47°C and 51°C. At 47°C (Figure 2) a shoulder similar to that noted with irradiation alone was observed, but

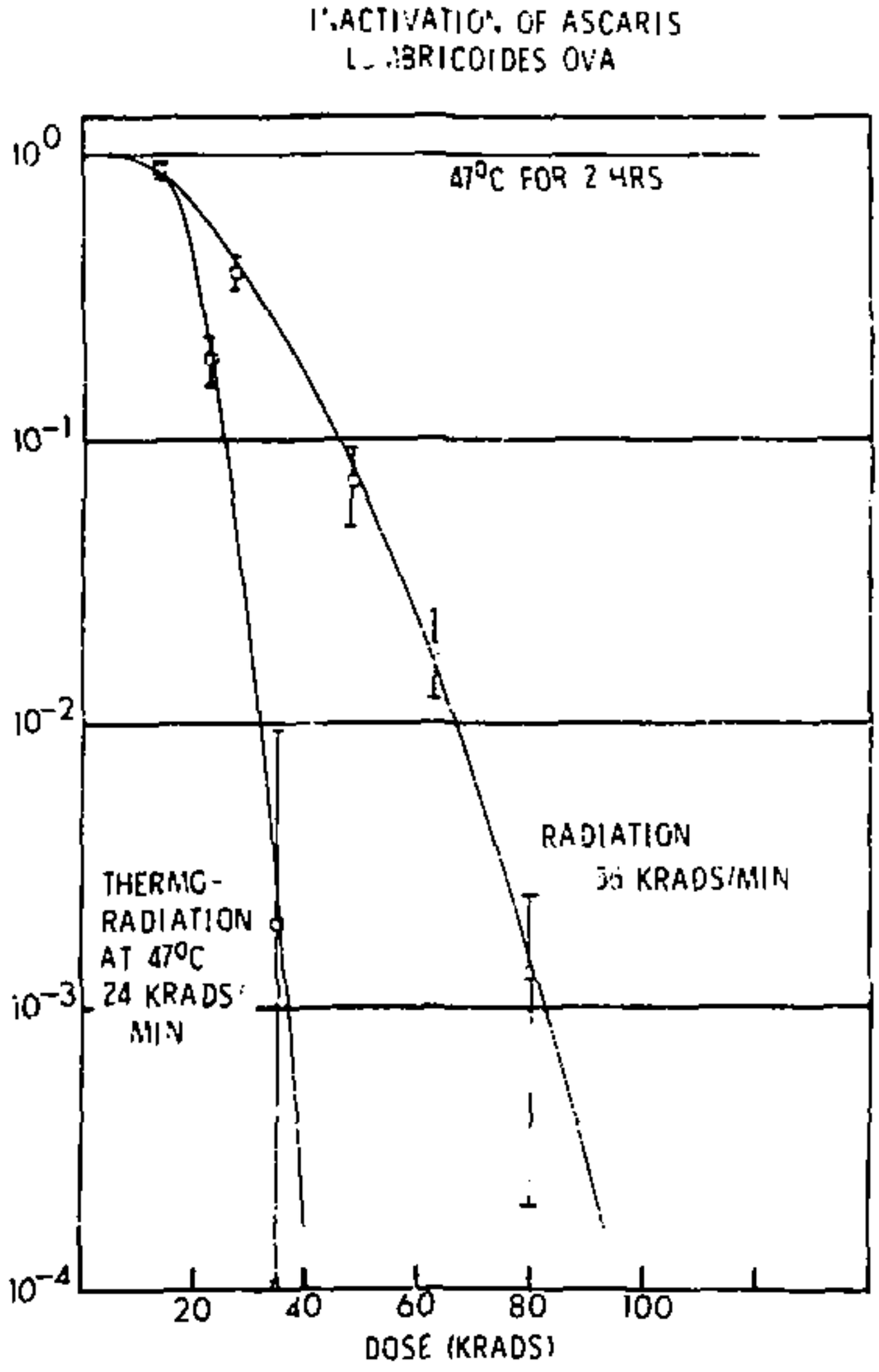

Figure 2. Synergistic inactivation of Ascaris ova by thermoradiation. Bars represent 95% confidence intervals.

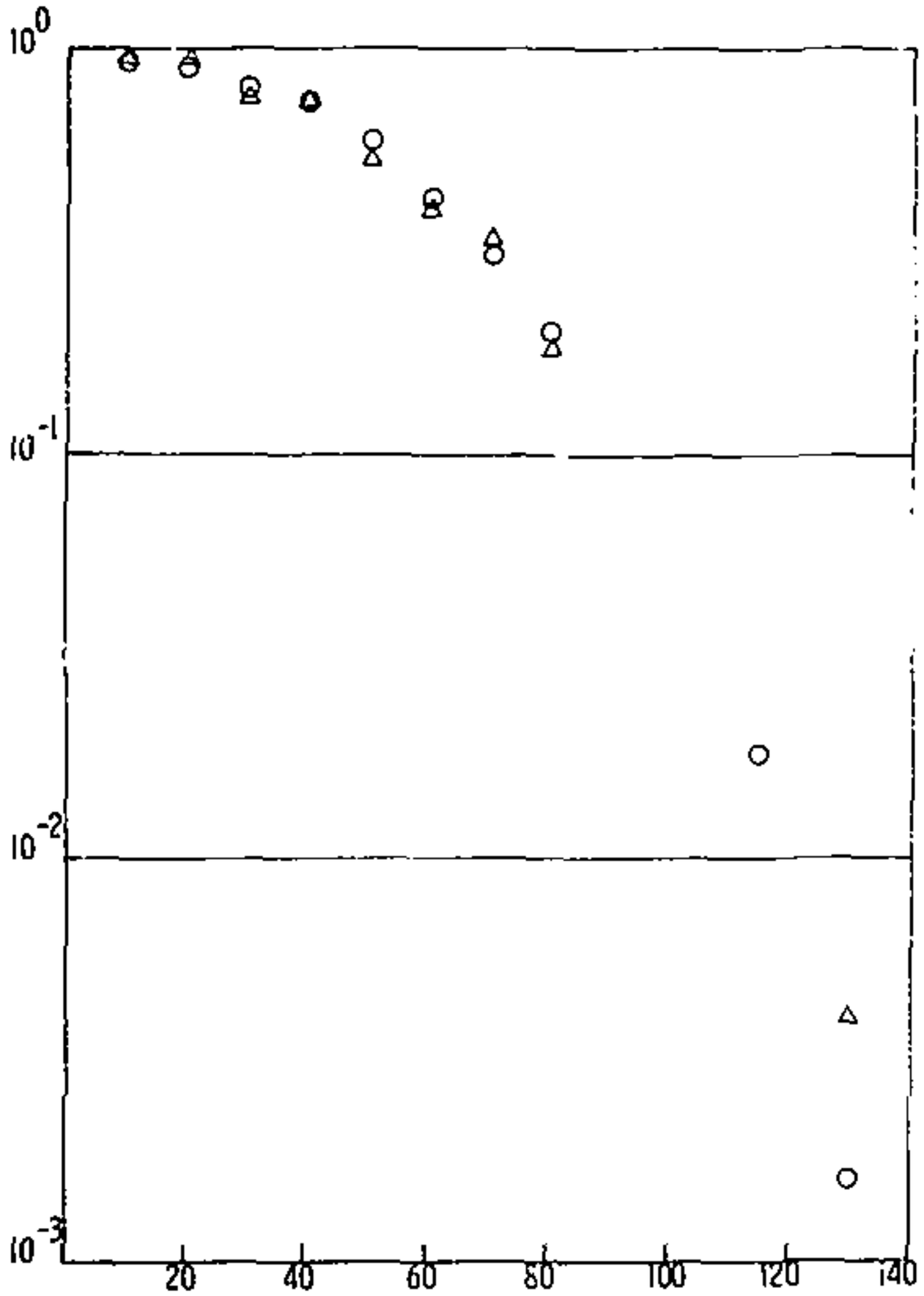

Figure 3. Inactivation of Ascaris ova by irradiation in sewage sludge, △, as compared to water, ○.

with additional irradiation, embryonation decreased rapidly as follows:

1st log decrease - 26 krads

2nd log decrease - 6 krads

3rd log decrease - 5 krads

No embryonated ova were observed at a total dose of 45 krads and only between 0.2 and 1.5% at a dose of 37 krads.

The results from 51°C thermoradiation experiments (Figure 4) were very similar to the 47°C results for the ova irradiated in a water suspension. At 60°C, embryonation after exposure to 10 krads was less than 0.1% with total inactivation at 15 krads. At 60°C, after exposure to 10 krads, embryonation was less than 0.1% with total inactivation at ≤15 krads. A difference was noted, however, when the ova were exposed in a sludge suspension. For example, it was noted that in sludge a three log reduction in embryonation required 65 krads as compared to about 40 krads for exposure in water.

DISCUSSION

Preparation of Ova for Storage or Use

A number of experimenters have developed their own protocol for extracting and cleaning Ascaris lumbricoides ova for subsequent experimentation (10, 11, 12). This experimentation required large quantities of fertilized eggs, therefore procedures which provided the greatest yield in the most efficient manner were selected.

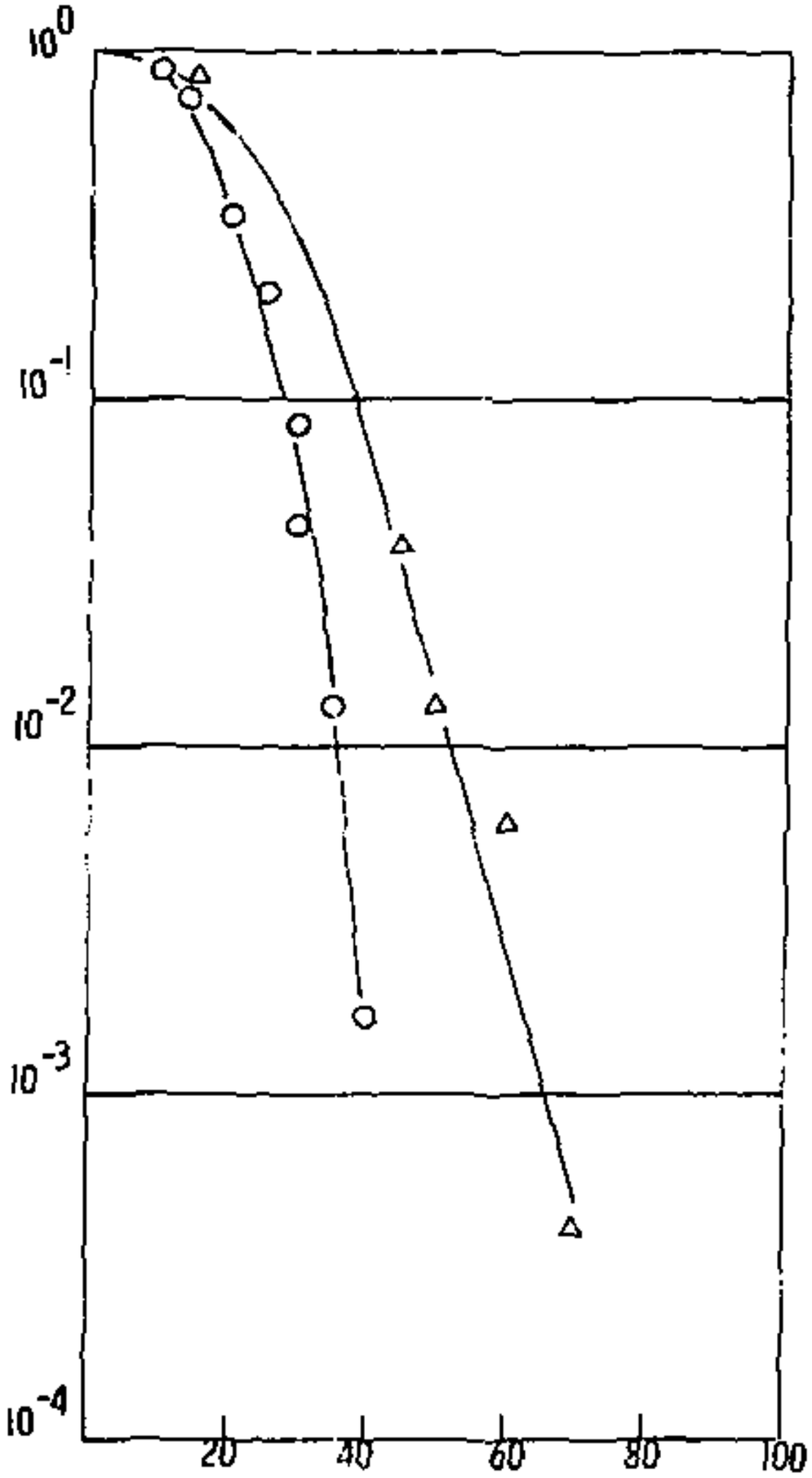

Figure 4. Thermoradiative inactivation of Ascaris ova at 51°C in sewage sludge, Δ, as compared to water, O.

It was determined that about 5 cm segments of the bifurcate uterus would provide the greatest quantity of ova, most of which were fertilized. This represented a compromise between the 1-2 cm used by Titani, et al (13) and the 8 cm used by Fairbairn (11). In this way, the percent of non-fertilized ova to the total count was held to 6-8%. Also, from this length segment of uteri, it was found that the control samples showed that an average of about 93% of the fertilized ova completely embryonated to the larval stage. The yield of fertilized ova per worm varied and ranged from 6.1×10^4 to 5.5×10^5.

Even though the proteinaceous layer was virtually removed by the method described, we experienced ova losses through their adherence to laboratory glassware surfaces. For this reason, several steps were taken which minimized the loss of eggs. The use of a blender to chop the uteri into small pieces was discarded in favor of the glass rod method. Similarly, separation of ova from supernatant after washings was accomplished by settling rather than centrifugation. The ova settled readily (15-20 minutes) in deionized water and in 0.1N H_2SO_4, but required a longer time (30-40 minutes) in NaOH. Whenever possible, plastic disposable pipets were used in place of glass pipets, which tended to reduce ova loss. Also, a silicon coating was applied to certain pieces of glassware. This was reasonably effective, but could not be used during the first two washes because the NaOH would have removed the coating.

In order to establish base line data against which the effects of thermoradiation could be compared, it was first necessary to examine the separate effects of exposing Ascaris lumbricoides ova to heat and irradia-

tion environments. In this way inactivation parameters were determined for various temperatures and radiation doses.

Heat

The results obtained from the experiments conducted in this study indicate a narrow temperature band in which temperature effects vary from no inhibition of embryonation at 47°C to a very sharp drop and nearly complete inhibition at 55°C for a 4-minute exposure. Experiments conducted at 51°C suggest this temperature to be the threshold at which heat effects become readily discernable since no inhibition was observed after 50-70 minutes at temperatures below 51°C. Exposure to temperatures in excess of 51°C showed an increasing inactivation rate. For example, < 1% of the ova exposed to 64°C for one minuted embryonated.

During the initial heat experiments, some spurious results in the form of a tailing effect were encountered where generally < 1% of the ova counted were embryonated even after extended periods of heat exposure. We theorized that these embryonated ova were the result of ova sticking to the sides of the tubes above the internal water line and therefore not subjected to the degree of heat experienced by the ova in the water suspension. This situation was resolved by flaming the upper portion of the tube, above the water line, while the lower portion was cooling in ice water for 30 seconds, subsequent to heat exposure.

An experiment was conducted at 51°C to determine whether the substitution of 1 ml of sludge for 1 ml of water (total of 5 ml in tube) would affect the results of heat exposure. Survival (embryonation) was slightly higher for the ova heated in the sludge suspension, but the difference was considered insignificant.

Irradiation

Ascaris ova are known to be sensitive to irradiation (13), although low doses of gamma irradiation up to about 15 krads did not show any appreciable effect on embryonation, as depicted by the small shoulder in Figure 2. As the dose increased, the irradiation effect became readily apparent. It was evident, however, that the shouldering effect continued to influence the inactivation rate, as each successive log drop in embryonation required less total dose.

We found it difficult to compare our results with others so far as irradiation effects on *Ascaris* ova are concerned, for the following reasons:

1. Some dose measurements are expressed in absorbed dose while others designate exposure dose, as evidenced by the use of the terms rep, rad, and roentgen.
2. In some cases, the dose rate was not given and in others it was as low as 0.11 krads/min., compared to the dose rates of about 18 to 36 krads/min. used in most of our radiation experiments.
3. Most researchers conduct their experiments with a specific purpose in mind, i.e., the effect of various doses of irradiation or the effect of irradiation at various stages of embryonation rather than complete suppression of embryonation, and therefore the results are not easily comparable.

In several irradiation experiments, it was noted that ova exposed to doses of > 100 krads had advanced to 2-, 4-, and 8-cell stages during incubation for 21 days but none had embryonated to the larval stage. In two cases, the ova suspension was incubated for an additional 10 days. In neither case were fully embryonated ova observed after 31 days incu-

bation, thus confirming the findings of Yanga (), that irradiation [illegible] is irreversible.

Thermoradiation

The effectiveness of thermoradiation has been established for inactivation of Bacillus subtilis var. niger (19). As described earlier, exposure of Ascaris ova to either heat or irradiation environments established thresholds which inhibit embryonation. Since the purpose of this study was to determine the lowest levels of heat and irradiation needed to completely inhibit embryonation, we also investigated thermoradiation in order to take advantage of the synergistic effect often found in the inactivation of biological systems.

A temperature of 47°C was selected for initial thermoradiation experiments because this temperature was slightly below the most effective [illegible]. Therefore, any decrease in inactivation beyond that due to radiation alone can be attributed to the synergistic effects of thermoradiation.

As shown in Figure 2, thermoradiation at 47°C displays a significant synergistic effect. The time [illegible] required to suppress embryonation was less than half that needed [illegible]. One might expect a slightly faster inactivation rate at 47°C, but this was not the case. The results at 47°C were nearly the same as those at 45°C. This situation might be explained by the fact that at what effects are evident for the first 15 minutes exposure at 47°C the thermoradiation exposure time was only slightly more than 1 minute. [illegible], experiments at 45°C and 47°C were conducted using different [illegible] stages. Therefore, it is not surprising that the 45°C and 47°C results

are very similar, especially when a difference in ambient temperature radiation resistance is noted (compare Figure 2 and Figure 3).

It was noted that inactivation occurred at a somewhat lower rate during thermoradiation in a sludge suspension than in a water suspension (Figure 4). The total dose required for extinction was ~75 krads at 51°C which is well within the estimated system (6) operating limits of about 200 krads at 60°C, especially since we found total extinction in water after 45 seconds and 15 krads at 60°C.

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