Demonstration of a Toxic Factor(s) in Thermal Death.* (22342)

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Although fluid balance may play an important role in the problem of shock and thermal death, other investigations have supported the theory that toxic substances are released into the circulatory system after scalding and that this toxic material is responsible for death of the organism(1-3). Helderweirt *et al.*(4), experimenting with heat death in rabbits, noted that even when lowered blood volume of scalded rabbits is experimentally returned to normal, the organism succumbs to "another trouble still unknown." The work of LaFontaine(5) and Simonart(6,7) indicates the importance of a toxic factor in blood of scalded frogs. Although the precise function of toxic substances is still argued, it has been suggested that the toxic factor is in some way connected with protoplasmic and blood clotting(8-10).

The arguments for and against the toxic factor theory have, however, been based on experiments involving only vertebrate organisms. It is, therefore, the purpose of this study to determine if a toxic substance could be found in marine invertebrates. Although preliminary experiments uncovered several invertebrate organisms which appeared to release a toxic substance when scalded (for example, starfish), this paper will deal solely with an investigation on the marine worm, *Phascolosoma gouldii.* One can easily obtain fairly large quantities of coelomic fluid from this thick-walled, unsegmented annelid. Another advantage in using this animal is that it does not undergo autotamy when subjected to scalding, a problem frequently encountered when experimenting with many other invertebrates.

Materials and methods. In the experiments to be described, both male and female worms *(Phascolosoma gouldii)* ranging from 3-5 inches in length were used. When scalding was necessary, the posterior half of the animal was immersed for 90 seconds in hot sea water (76 ± 1°C). Following this treatment the animals were immediately returned to finger bowls containing sea water and maintained at a relatively constant temperature by stacking the bowls on sea water tables. The water within the finger bowls was changed at least once daily. In those experiments involving dialysis, dialysis membrane having a flat diameter of 1 1/16 inches was washed both inside and out, and the samples dialyzed against large volumes of running sea water (approximately 500 ml per minute) for 12 hours. The end point used in determining the survival time of worms is probably subject to an error of at least ± 2-3 hours. These organisms were considered dead when pinching them with a forceps no longer produced a response such as muscular contractions. Unless otherwise stated, in all experiments a minimum of 15 animals was used to determine average survival time. Eggs of the sea urchin, *Arbacia punctulata,* were obtained by electrical stimulation, as described by Harvey(11). The eggs were washed several times in sea water and fertilized by addition of diluted sperm. The per cent cleavage was determined by counting a minimum of 100 eggs per dish.

Results. Suggestive data for the release of a toxic substance was first obtained when comparing the survival time of scalded worms to that of worms ligated prior to scalding. Results indicated that, although scalded animals would die in about 21 hours, the survival time could be increased merely by securing a ligature (a heavy thread or Hoffman clamp) just anterior to the heated region, prior to scalding (Table I). It can be seen that by preventing the coelomic fluid in the scalded portion of the animal from mixing with the anterior portion, the survival time was increased. The fact that survival time could

* Supported in part by grants from the Coe Research Fund.

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TABLE I. Effect of Pre-Scalding Ligature on Average Survival Time (in Hr ± S.D.) of Scalded Worms. Posterior half of organism immersed in sea water at 76°C for 90 sec.

Non-constricted animal		Animal constricted by ligature	
Scalded	Control	Scalded	Control
21 ± 13	Infinite	74 ± 29	120 ± 79

not be increased any longer than 3- or 4-fold may be explained on the basis of a leak in the ligature, but more likely by the fact that the procedure of ligating is in itself sufficient to cause death in 120 hours.

In an attempt to isolate any toxic material that was present in scalded worms, coelomic fluid was removed from worms 15 minutes after scalding by piercing the thick muscular wall with an 18 gauge hypodermic needle attached to a 5 ml syringe. The coelomic fluid was then centrifuged and 1 ml aliquots of the supernatant injected cephalad into the posterior end of normal recipient worms. As shown in Table II, coelomic fluid from scalded animals proved toxic when injected into normal recipients. The controls, when injected with 1 ml of cell-free coelomic fluid obtained from unheated *Phascolosoma,* lived indefinitely. It appears, then, that the act of scalding releases a toxic substance which is found in the coelomic fluid and is capable of causing death when injected into normal animals of the same species.

Origin of the toxic factor. Although the toxic substance is found in the coelomic fluid, the question arises as to whether the toxin is released from coelomic fluid itself or if it originates from another source. In an attempt to answer this question, experiments were carried out in which the coelomic fluid was removed from normal worms, the coelomic cavity flushed out (with sea water) and finally refilled with sea water. This "sea-water containing" worm was then scalded and the fluid within the coelomic cavity removed, centrifuged and 1 ml aliquots injected into normal recipient worms. The results obtained from this type of experiment showed that toxin was not released in those scalded animals where sea water was injected into the coelomic cavity in place of coelomic fluid. Such experiments suggest that the toxic substance originates in the coelomic fluid. However, the possibility still exists that, in order for the toxin to be released, an inter-action may be necessary between the coelomic fluid and the rest of the worm. In the following experiments the coelomic fluid from normal animals was removed, placed in test tubes and immersed in a water bath at 76°C for 90 seconds (2 minutes in instances where the diameter of the tubes was substantially larger than the diameter of worms). This heated coelomic fluid was cooled, centrifuged and 1 ml aliquots of the supernatant injected into normal worms. The results show that the toxic factor can be released merely by heating coelomic fluid *in vitro* since those animals receiving injections of this fluid succumb in about 40 hours. (Controls receiving 1 ml injections of unheated coelomic fluid lived indefinitely.) It appears, then that the toxin can be released without any interaction between coelomic fluid and the rest of the organism. Additional experiments were carried out in which pieces of body wall, as well as viscera, were heated *in vitro* in the presence of small volumes of sea water; but the supernatant, when injected into recipient worms, proved non-toxic.

TABLE II. Effect of Cell-Free Coelomic Fluid Taken from Scalded Worms on Average Survival Time (in Hr ± S.D.) of Normal Worms.

Worms inj. with coelomic fluid from scalded donor	Worms inj. with coelomic fluid from normal donor
29 ± 15	Infinite

By separating coelomic fluid into two portions, (1) the cells of the coelomic fluid—which were re-suspended in sea water and (2) the cell-free supernatant, it was shown, after heating at 76°C, that the toxic factor was released only in that fraction of coelomic fluid containing the cells. Thus the origin of the toxic substance released in this marine annelid after scalding appears to be in the cells of the coelomic fluid. It might be brought out at this point that preliminary experiments suggest that the toxin can be released by treatment other than heat—particularly homogenization.

Characteristics of toxic factor. The series of results, tabulated in Table III, show that

TABLE III. Effect of Heat and Dialysis on Toxic Factor Activity. Average survival time (in hr ± S.D.) of normal worms receiving 1 ml injections of precipitate-free coelomic fluid.

Cell-free coelomic fluid of scalded worm		Cell-free coelomic fluid of normal worm	
Autoclaved	Autoclaved & dialyzed	Autoclaved	Autoclaved & dialyzed
32 ± 14*	77 ± 32	Infinite*	Infinite

* Only 9 worms injected.

the toxic factor is still active after autoclaving (15 lb pressure per square inch). This heat-stable toxin appears to be a relatively large molecule (or at least tied up with a large molecule) in that after prolonged dialysis against running sea water, the toxic fraction still proves fatal when injected into normal recipients (Table III).

Experiments have shown that the toxic factor was not ether-soluble since ether extracts of toxic coelomic fluid, after having been evaporated and redissolved in sea water, had no apparent effect when injected into normal worms. The ether-insoluble portion still exhibited its toxicity. Although the toxic coelomic fluid has a lower pH than does the normal (normal = pH 7.8; toxic = 6.7), readjusting the hydrogen ion concentration, followed by injection of normal worms, shows that the toxicity was not due to any pH change. In like manner, it was shown that the toxicity was not due to any potassium differences that may be present between experimental and control coelomic fluids.

In an attempt to further isolate the toxic factor, samples of the coelomic fluid were heated *in vitro,* centrifuged and the supernatant autoclaved. The protein coagulated by autoclaving was then centrifuged off and the supernatant then saturated with ammonium sulfate. This resulted in the appearance of a flocculent precipitate. These fractions were separated by centrifugation and the precipitate redissolved in sea water (a volume equal to that of the supernatant). The 2 fractions, the supernatant and the precipitate, were then dialyzed until all traces of ammonium sulfate had been removed. When 1 ml aliquots of these fractions were injected into normal worms (Table IV) the fraction containing the precipitate, which was "salted out" by saturation with ammonium sulfate, proved toxic.

It might be noticed, when comparing the data presented in Tables II, III and IV, that animals injected with samples of coelomic fluid that have been dialyzed survive over twice as long as those animals injected with non-dialyzed coelomic fluid. These data suggest the possibility that two types of toxic molecules are released after scalding—one dialyzable and the other non-dialyzable.

Semi-micro assay. Since the only method of assay has been that of injecting fluid into recipient worms, a procedure requiring large volumes of toxin, it was felt that a micro assay was necessary, particularly if chromatographic studies were to be pursued. Eggs of the sea urchin, *Arbacia punctulata,* are relatively sensitive to the toxic factor, for when these eggs were fertilized in sea water and 5 minutes later immersed in the toxic fraction, cell division was substantially inhibited. It has been shown that only 6% of the eggs undergo cleavage when immersed in a 6.25% solution of cell-free coelomic fluid taken from scalded worms (diluted in sea water). Further proof that the factor inhibiting cell division is the same factor responsible for the toxic effects in the worm is seen in experiments in which fertilized *Arbacia* eggs were immersed in the fraction precipitated by ammonium sulfate (similar to those used in Table IV). As can be seen in Table V, cell division was completely inhibited in such instances although the various controls underwent substantial cleavage.

Summary. In a study of heat death, it has been shown that the marine annelid, *Phascolosoma gouldii,* releases a heat stable, non-

TABLE IV. Isolation of Toxic Factor by "Salting Out" with $(NH_4)_2SO_4$. Assayed by determining average survival time (in hr ± S.D.) of worms receiving 1 ml injections. Coelomic fluids were partially purified prior to saturation with $(NH_4)_2SO_4$ by autoclaving.

Partially purified toxic coelomic fluid saturated with $(NH_4)_2SO_4$		Partially purified normal coelomic fluid saturated with $(NH_4)_2SO_4$	
Precipitate (dialyzed)	Supernatant (dialyzed)	Precipitate (dialyzed)	Supernatant (dialyzed)
56 ± 13	Infinite	Infinite*	Infinite

* Only 8 worms injected.

TABLE V. Effect of Toxic Factor, Isolated by "Salting Out" with $(NH_4)_2SO_4$, on Cleavage of *Arbacia punctulata* Eggs. Coelomic fluids were partially purified by autoclaving, followed by centrifugation and dialysis.

	Partially purified toxic coelomic fluid saturated with $(NH_4)_2SO_4$		Partially purified normal coelomic fluid saturated with $(NH_4)_2SO_4$		
	Precipitate	Supernatant	Precipitate	Supernatant	Sea water control
	(Dialyzed)				
% of eggs cleaved	0	97	94	96	99

dialyzable toxic factor, capable of causing death when injected into normal worms. This toxic substance, originated from the cells of the coelomic fluid, has been partially isolated by saturation with ammonium sulfate. A semi-microbiological assay has been described.

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Received February 20, 1956. P.S.E.B.M., 1956, v91.

Reduction of Mitotic Activity in Pinna Epidermis of Mice Given Cortisol or 9α-fluorocortisol.*† (22343)

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The inhibitory effect of cortisone upon mitotic activity in certain tissues seems to be established(1-4). In this study, antimitotic effects were noted for hydrocortisone (cortisol) and for 9α-fluorocortisol. In order to detect antimitotic effects occurring within the range of the daily mitotic rhythm, this work was carried out 1) under environmental conditions standardized for evaluation of 24-hour periodicity(5), 2) during the period of day of relatively high mitotic activity, 3) on a population of mice previously found to exhibit a marked mitotic rhythm in pinna epidermis(6).

Materials and methods. For 7 days prior to experiment, male ZBC mice, 8 weeks of age, were kept singly housed in a room maintained at 80 ± 1°F, illuminated from 6 A.M. to 6 P.M., and kept dark from 6 P.M. to 6 A.M. Purina Fox Chow and tap water were available to the mice *ad libitum*, from the time of weaning and throughout experiment. On the day of experiment, a first group of 11 mice was not handled, while 3 other groups, each composed of 12 mice, were given intraperitoneally 100 γ or 1,000 γ of cortisol acetate or 10 γ of 9α-fluorocortisol acetate. Each mouse was weighed prior to injection and the dose was adjusted to refer to 20 g of body weight. The treatments were given in rotation, in order to minimize the possible role of a time factor difference among treatments.

* This investigation was supported in part by the Elsa U. Pardee Foundation and by research grants from Amer. Cancer Society, the U. S. Public Health Service (G-4158) and the Graduate School of University of Minnesota.

† The authors are indebted to Dean William H. Crawford, University of Minnesota Dental School for facilities of laboratory and to Dr. L. H. Sarett and Dr. E. Alpert of Merck & Company, Rahway, N. J., for gifts of steroids.