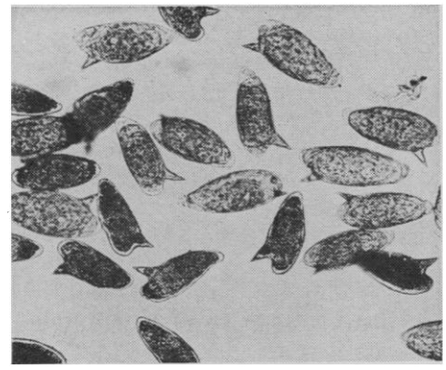
Schistosome Ova: Rapid Separation from Mouse Tissue by Digestion with Pinguinain

Abstract. *High yields of ova of* Schistosoma mansoni *were obtained from the livers and intestines of infected mice by digestion of the homogenized tissue with the proteolytic enzyme pinguinain.*

Studies of the ova of *Schistosoma mansoni* have been somewhat hampered by the lack of a simple, reliable method for the bulk isolation of the eggs from the liver and intestines of infected mice. The methods of Coker and Lichtenberg (*1*) and Ritchie and Berríos-Durán (*2*), based mainly on sedimentation and decantation, are useful for separating eggs in small amounts from liver tissue. The method developed by Smithers (*3*), based on tissue digestion, requires the use of two of the proteolytic enzymes, trypsin and pepsin, and the incubation period of the liver homogenates extends over 5 hours. Both enzymes show peptide bond specificity and have different *p*H optima, so that incubation in two different buffers is required to achieve maximum hydrolysis. Commercial preparations of these enzymes are expensive. Recently, Browne and Thomas (*4*) developed an enzymatic method for obtaining viable ova from the liver of hamsters. Their method, although complex and requiring special apparatus, yields ova suitable for tissue culture studies.

We have now developed a rapid enzymatic method of separating Schistosome ova from the liver and intestines of mice in quantities sufficient for the circumoval precipitin test (*5*) to be conducted on a large scale, and for us to be able to obtain relatively large amounts of the antigenic substance present in the ova.

Mice were obtained (*6*) which previously had been infected with *Schistosoma mansoni*, according to the method described by Ritchie and Berríos-Durán (*2*). The mice were killed and the livers and intestines removed. Whole (average weight 3 g) or homogenized livers, or washed intestines (average weight 2.5 g) were placed together with 0.1*M* acetate buffer (2 ml/g of tissue, *p*H 4.0) in a diagonally fluted pyrex flask (capacity 5 to 30 ml) and were homogenized with a Virtis 23 homogenizer. The equivalent of two homogenized livers or two intestines were transferred into 25-ml Erlenmeyer flasks. A crude preparation of pinguinain (*7*), a very active proteolytic enzyme, was then added to the homogenate at the rate of 5 to 10 mg/g of tissue. The flasks were placed in a water bath maintained at 37°C and constantly shaken for 3 hours. Undigested fatty material adhered to the walls of the container, while free eggs sedimented to the bottom. After digestion, the contents of each flask were passed through a fine mesh (15/cm²; 100/in²). A layer of 6 ml of the filtrate thus obtained was placed over 8 ml of an 8-percent sodium chloride solution (wt./vol.), in a 15-ml graduated tube, and was then centrifuged for about 2 minutes at 1000 rev/min in a table centrifuge. The eggs sedimented to the bottom. Debris remaining in the upper layer was removed by suction and the remaining sodium chloride solution was decanted. The sedimented eggs were again suspended in a solution of sodium chloride (0.9 percent), layered over 8 ml of an 8 percent sodium chloride solution, and centrifuged. By this procedure, which, after incubation, took about 15 minutes, we obtained 2000 to 3000 eggs per gram of liver, and 8000 to 10,000 eggs per gram of intestine.



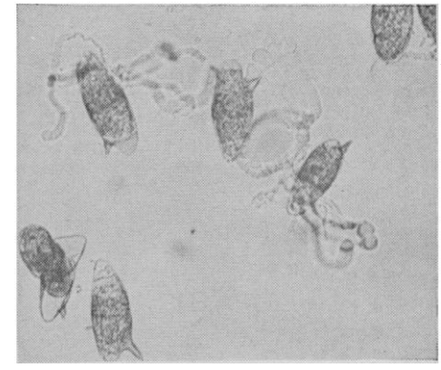


Fig. 1 (left). Ova of *S. mansoni* obtained from mouse liver by incubation with pinguinain (× 170). Fig. 2 (right). Circumoval precipitate observed after incubating ova of *S. mansoni* with immune human serum for 24 hours (× 170).

The eggs were completely free of debris (Fig. 1). Under the microscope the miracidia appeared to be physically intact, but they were immotile, and we were unable to make them hatch. Positive circumoval reactions were observed when the fresh or lyophilized ova were incubated with sera of patients with schistosomiasis (Fig. 2). In addition, the ova separated by this method yielded a positive circumoval reaction after 2 hours of incubation, a period considerably shorter than is usually required to obtain a positive test (*8*).

Apparently, the low *p*H of the acetate buffer used during digestion affected the motility of miracidia. Although we did not obtain living miracidia, and in spite of the possibility of changes having occurred in the antigenicity of the egg membrane, this method has several advantages. It is simple and rapid, and it enables high yields of ova to be obtained both from the liver and from the intestines. Our method overcomes many of the difficulties in isolating reactive ova, which have previously limited the use of the circumoval precipitin test as a routine clinical tool in the diagnosis of schistosomiasis (*9, 10*).

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References and Notes

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6. Many of these were kindly supplied by the U.S. Army Tropical Research Medical Laboratory, Parasitology Section, Fort Brooke, Puerto Rico.
7. Pinguinain is a proteolytic enzyme, dependent on its sulfhydril groups for enzymatic activity, and showing a broad peptide bond specificity. It is present in appreciable amounts in the fruits of *Bromelia pinguin* L. The pinguinain used in this research was prepared in our laboratory. C. F. Asenjo and M. del C. Fernández, *Science* **95**, 48 (1942); R. A. Messing, A. F. Santoro, A. Bloch, *Enzymologia* **22**, 110 (1960); E. Toro-Goyco, M. Matos, M. Cancio, *Federation Proc.* **22**, 528 (1963).
8. A definite circumoval precipitate could be observed under the microscope after 2 hours' incubation. Serums which gave a 4+ reaction after 24 hours' incubation gave a 1+ reaction as early as 30 minutes after incubation. Furthermore, the percentage of ova which gave a positive circumoval test

when incubated with 4+ sera (40 percent) was higher than given by ova separated by methods based on sedimentation and decantation (20 percent or less).

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Plague Toxin: Its Effect in vitro and in vivo

Abstract. *The murine toxin of* Pasteurella pestis *inhibited the respiration of heart mitochondria from the rat and the mouse but had little or no effect on the respiration of mitochondria from the rabbit, chimpanzee, dog, and monkey. Alterations occurred in the S-T segments of the electrocardiograms recorded from rats injected with ¼ to 10 LD_{50} of toxin, but not in those from rats dying of hemorrhagic shock, hypoxia, intoxication with glucose, or* Escherichia coli *endotoxin. No abnormalities were observed in electrocardiograms from rabbits injected with large amounts of toxin.*

Purified murine toxin from *Pasteurella pestis* is lethal to mice and rats, but not to rabbits (*1*). It has also been found that the respiration (in terms of oxygen consumption) of mitochondria obtained from the heart of the toxin-sensitive rat is inhibited by the toxin, whereas heart mitochondria from the toxin-resistant rabbit are unaffected. It was our purpose to study further the specificity of the effect of the toxin on animals other than the rat, mouse and rabbit, and to correlate the observations in vitro with a number of experiments in vivo based on electrocardiographic measurements of intoxicated and unaffected animals.

Pasteurella pestis, strain TJW, was grown under conditions described previously (*2*). The toxin, which was prepared and purified as before (*2*), had an intraperitoneal LD_{50} for albino Swiss mice of 45 to 56 μg toxin/kg body weight (calculated according to the method of Reed and Muench) (*3*).

Heart mitochondria were isolated according to the method of Cleland and Slater (*4*), and oxygen consumption was measured by means of a sensitive polarographic method, according to the procedure outlined by Packer *et al.* (*1*). Protein concentration was determined by a modified Lowry method described by Oyama and Eagle (*5*).

The rats were anesthetized with nembutal when electrocardiographic measurements were made. Blood pressure was measured with a mercury manometer by cannulating an exposed femoral artery. Needle electrodes were inserted into the four legs of the rats and connected to an upright Sanborn electrocardiograph. After the blood pressure stabilized following the surgical procedures, a standard three-lead electrocardiogram was recorded as a baseline. Toxin was then inoculated (¼ LD_{50} to 10 LD_{50}) and electrocardiographic tracings were obtained, as well as hematocrit and blood pressure measurements.

Hemorrhagic shock was induced in the animals by severing the femoral artery; they were intoxicated with glucose by injecting intraperitoneally 5 to 10 ml of a 50-percent glucose solution; hypoxia was brought about by placing a plastic bag over the head of the animal.

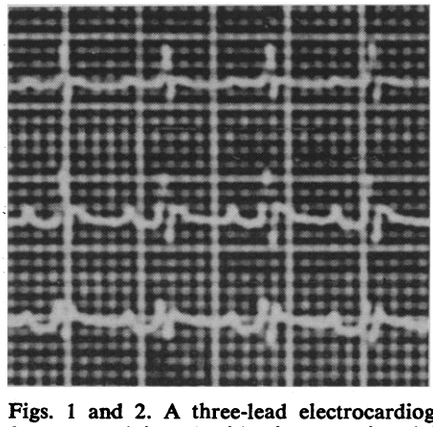
The chimpanzee, dog, and monkey remained completely unaffected when injected with amounts of toxin that represented 50 to 100 times the mouse LD_{50} on a kg body weight basis. Experiments in vitro have shown that the respiration of the heart mitochondria of these animals was likewise unaffected, as shown in Table 1. With α-ketoglutarate as substrate, significant inhibition was apparent only with mitochondria from the rat and mouse heart and not with those obtained from the monkey, dog, rabbit, or chimpanzee.

Table 1. Effect of plague toxin on the respiration of heart mitochondria from the rat, mouse, monkey, dog, rabbit, and chimpanzee. The reaction mixture in the cuvette (1.0 ml) consisted of mitochondrial protein (1.69 mg for the rat and mouse, 1.39 mg for the other animals); 0.32*M* sucrose, 0.01*M* KCl, 0.001*M* disodium salt of ethylenediaminetetraacetic acid, 0.02*M* phosphate buffer; the *p*H was 7.5, and the temperature, 26°C. Plague toxin was incubated with the reaction mixture for 3 minutes before testing respiration; the control was incubated without toxin. Respiration was allowed to continue for 90 to 120 seconds and then α-ketoglutarate was added to give a final 10 m*M* concentration.

Source of mitochondria	O_2 consumed (μmole/lit. sec)		Inhibition (%)
	No toxin	Toxin (2.5 mg)	
Rat	0.84	0.53	37
Mouse	0.60	0.44	26
Monkey	0.70	0.70	0
Dog	0.64	0.62	4
Rabbit	0.43	0.41	5
Chimpanzee	0.48	0.52	0

Following these experiments, attempts were made to correlate the changes in vitro with the results of the experiments in vivo. It was assumed that since mitochondria from the rat heart were susceptible to the action of the murine toxin of *Pasteurella pestis* alterations in the myocardial physiology of the rat might be expected which could possibly be detected by electrocardiographic measurements. The results are shown in Figs. 1 and 2. Alterations in the S-T segment of the electrocardiogram occurred within 60 minutes after the injection of ¼ to 10 LD_{50} of toxin, and prior to changes in hematocrit or blood pressure measurements. It was interesting to note that in surviving animals that received ¼ LD_{50} of toxin, the initial changes in the electrocardiograms were no longer evident after 24 to 48 hours, or after the animal had completely recovered.

Similar changes did not occur in rats dying from hemorrhagic shock, hypoxia, or intoxication with glucose or *Escherichia coli* endotoxin. Electrocardiograms from the rabbit, which is resistant to murine toxin, did not show any abnormality, even when the toxin



Figs. 1 and 2. A three-lead electrocardiogram recorded from a normal rat (left) and from a rat injected with plague toxin (right).