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Studies on Excystment of Clinostomum Metacercariae by Use of Artificial Digestion*

MARILYN HEMENWAY

INTRODUCTION

For many yaers parasitologists have studied a group of animals called flukes. Although much is known about them now, difficulty has been encountered in obtaining the metacercarial form for study. At present there is no universally satisfactory method available to free metacercariae from the cyst walls that enclose them. The development of such a method would remove a stumbling block from the path of further study of the fluke life histories.

A metacercaria is a stage in the life cycle of a fluke, a parasitic flatworm of the Class Trematoda. Trematodes that are internal parasites have extremely complicated life histories, and Clinostomum, the cysts of which were used in the following experiments, is no exception. In water the eggs of the fluke hatch into miracidia which penetrate into several species of snails. In the liver of the snail are sporocysts which develop rediae. The rediae give rise to cercariae which leave the snail as free-swimming larvae and penetrate tadpoles. In the tadpole a cercaria migrates to the body cavity and encysts on the mesenteries of the host by forming a membrane around itself. A second, outer membrane is later formed, presumably by the host.[1,2]

During this encysted or metacercarial stage, further development occurs, but the adult stage is reached only in the definitive host, a heron. When an infected tadpole or frog is eaten by a haron, the metacercaria excysts in the digestive tract, matures to adult stage, and lays eggs which are returned to water in the excreta of the heron.

Under natural conditions metacercariae excyst while in contact with definite physiological processes inside the definitive host. In the stomach gastric juice is secreted which contains, among other things, pepsin and HCl. The cyst wall breakdown starts when pepsin, a protein-splitting enzyme, is activated in the presence of HCl. Proteoses and peptones result as protein molecules are split by hydrolysis. The churning of the stomach agitates all contents. When the stomach contents pass into the small intestine one to four hours later, juices alkaline in reaction quickly mix in and neutralize acids. Part of this juice is from the pancreas, and it contains trypsin, an

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Experiments were carried out under the guidance of Mr. Parsons, instructor in biology at Grinnell College. He suggested methods of procedure and identified material for me when I was unable to do so. Dr. Oelke of the chemistry department donated a portion of his time to help me in pH determinations.

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enzyme which continues protein digestion by breaking proteoses and peptones into polypeptids, then tri- and dipeptids, and occasionally into the simple amino acids.[3] Constant temperature, usually about 37°C., is maintained throughout. Other enzymes, secretions, and partly digested materials are also present.

Experiments have indicated that pepsin works best in HCl solutions of .3% (pH 4).[4] In the presence of pepsin, globulin, a protein molecule, splits into two smaller fragments at a pH of 4.5 — 3, and, as the pH is lowered, still smaller fragments appear.[5] Trypsin works best in slightly alkaline mixtures, but it will carry out proteid digestion in neutral or .012% HCl. There are cases when the acidity of the stomach's contents are not neutralized until far along in the small intestine.[6] It has been suggested that if trypsin follows pepsin treatment, digestion is faster.[7]

In the past it has been taken for granted that cyst walls have been simply digested away while the fluke remained passive. Apparently this is not strictly true. Efforts have been made to obtain excysted metacercariae by other means than by outright dissection although Mr. Thomas Parsons and I could find little published on methods used. M. S. Ferguson has used an acidified and aqueous crude pepsin solution at 37°C. to free *Posthodiplostomum minimum*.[8] Mr. Parsons' work along the same line proved unsuccessful with Clinostomum and several other species.

Since there has been a high mortality of metacercariae when artificial means are used to free them, some safer method ought to be devised. Clues from actual physiological processes seemed to point the way towards a more applicable method. This study was made to try to duplicate, as near as possible, natural conditions and to discover their effect on Clinostomum metacercariae.

Clinostomum cysts were used for this study because they are quite commonly found in frogs which are easy to keep alive in the laboratory. The cysts were .716 mm. by .537 mm. on the average. The clear outer wall measured from .130 mm. to .150 mm. in depth. The rest of the cyst was light yellow in color. The inner wall depth was not discernible at this point. The inner cysts were .456 mm. by .256 mm. on the average. The cysts were found easily in an opened frog, for they stood out in the clear mesenteries against the darker organs. Most were found near the heart or near the pyloric region.

MATERIALS AND METHODS USED

All experiments were carried out in a constant temperature bath. An aquarium served as a water container in which a thermostatically controlled heating unit was suspended to keep the temperature at 37°C. A piece of glass tubing connected to an aerating unit by rubber tubing kept the water in circulation, and pipettes attached similarly to the same aerating unit served to agitate the contents of the test tubes containing experimental mixtures suspended in the bath. The rubber tubes were partially clamped off with screw clamps so that

Hemenway: Studies on Excystment of Clinostomum Metacercariae by Use of Arti

the amount of air passing through could be controlled. A thermometer was needed to check the water bath temperature.

An HCl solution with a pH of 4 was made up, and 150, 300, and 450 mg. portions of pepsin and trypsin were weighed out. Cysts were placed in test tubes containing 15 c.c. of Hcl solution with one of the above amounts of pepsin. At first the treatment was continued over night, but the metacercariae died. Cysts could be checked at any time during or after pepsin treatment. Checking at intervals, I found healthy cysts with the first wall removed even when the time was cut down to one hour. One of the above amounts of trypsin was then added with some $NaHCO_3$. By using universal litmus paper and neutral red indicator, I first obtained a mixture with a pH of 8 when 230 mg. $NaHCO_3$ was added. This pH would again correspond to natural conditions, but later I learned that protein material causes error in pH measurement by indicator color, according to a note in an experiment sheet given to me by Dr. Oelke. Upon obtaining no results from the trypsin half of my experiments, I happened to use only half as much $NaHCO_3$ and found that the cyst wall removal proceeded more satisfactorily. I finally resorted to adding two drops of neutral red directly into the mixture in the test tube and added just enough $NaHCO_3$ to destroy the red color imparted by the indicator. Time in trypsin digest was also cut down from overnight to 10-15 minutes. The trypsin solution was always very milky, so the mixture had to be washed several times with warm water before any cysts or flukes could be located. Their protein make-up caused them to sink, so top portions could be poured off during washing and still leave the fluke material at the bottom of the test tube.

The first experiment was carried out in 25 c.c. $NaHCO_3$ solution (pH 8) and 500 mg. of trypsin. After 17 hours the one cyst tested was lost. In the second experiment 15 c.c. of the same solution and 300 mg. of trypsin was found to be a better proportion for the test tube to hold. Three cysts were tested, and after 19 hours one of them had lost the outer wall although the parasite itself was dead. Another cyst was left with a cloudy outer wall, and the third cyst was gone. Pepsin treatment preceded trypsin treatment in the third experiment. To 15 c.c. .3% HCl solution was added 300 mg. pepsin. After 17 hours 150 mg. trypsin was added with 230 mg. $NaHCO_3$. No live material was left after six hours.

The results of the rest of the experiments are to be found on Tables No. 1 and No. 2. For Exp. A (Table No. 1) a 2% pepsin solution was used. Although digestion had proceeded too far at the end of 17 hours, slight internal changes in shape and expansion seemed to have taken place. In Exp. B the same amounts of digests were used, but the cysts were removed from the pepsin after 1 hr. and 5 min. By then both cysts were minus the outer wall. The remaining wall was a white, vacuole-filled mass which seemed to contract at intervals. After an hour had passed, both cysts stood on one end. This phenomenon was often observed later and seemed to be due to the contractions which directed the vacuoles towards one end to make that end

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lighter. In such cases the wall at the bottom was left perfectly clear. A half hour in 2% trypsin resulted in the loss of the second wall of one specimen, but a fine membrane still restricted the fluke. The other specimen was lost.

Solutions of 3% pepsin and 1% trypsin were used in Exp. C. Stages of outer wall breakdown were observed here. A thin, shell-like covering apparently allows the digests to penetrate. The material inside softens and expands, cracking the already weakened shell. The shell breaks up and disappears, and sometimes the metacercaria is still completely surrounded by the soft, gelatin-like remains of the outer wall. As the soft material is slowly digested, the metacercaria, still in its inner wall, changes shape. Once a part of it is outside, that part swells until only a narrow tail is left inside. As soon as this is free, the cyst regains its oval shape. No metacercaria was seen going through all these stages, but the account was pieced together from seeing different stages. The freed fluke in this experiment was contracted in a membrane.

The same procedure was followed for Exps. D and E except that the time in trypsin was cut down. Here it was decided that 1% solutions of digest, though eventually effective, were too slow. Three per cent solutions did not act twice as fast as 2% solutions, so the latter were considered the most practical and convenient for the remainder of the experiments.

Before adding trypsin in Exp. F, I added two drops of indicator directly to the mixture. By the time half of the 230 mg. of $NaHCO_3$ was added, the indicator red had vanished. Due to the yellow tint of the mixure itself and to protein error, the change probably occurred before a pH of 6.8 (the turning point for neutral red) was reached. Running the experiment with a smaller amount of base, I obtained a perfectly normal excysted metacercaria. The next two tries also yielded normal metacercariae.

By this time I had learned that only .015 g. of $NaHCO_3$ was needed to turn the neutral red to yellow although any amount below .05 g. seemed to work as well. Checking the pH resulting from adding various amounts of $NaHCO_3$ to the digests on a Colman pH meter, I found the following:

gms. $NaHCO_3$	pH
.050	6.38
.075	6.40
.100	6.65
.500	7.10

Exp. I did free one metacercaria, but also another lost both walls although a membrane still remained. It was dead. The most successful experiment was obviously J, and Exp. K was not much less so even though many cysts were lost. After trypsin treatment the one metacercaria left was extremely active as it thrashed around inside the second cyst wall. It often pushed out the sides in large bulges. Meanwhile, one end of the cyst wall had caved in until only a thin membrane seemed to separate the fluke from the outside. This soon broke easily, and the fluke emerged. Being more active than the

one seen emerging in Exp. G, this one was out in about three minutes. The other excystment had taken 10-15 minutes.

The next experiment performed was worthless because the cysts had stood in water overnight. Only four of the fourteen came out of pepsin with the first walls removed. In the ten minute trypsin treatment .05 g. of $NaHCO_3$ were used, and all died. In the last experiment all but one was lost although this one was very active. Saving the very top layer from the test tube, I found two empty cyst walls; but the fine membranes around the open end had dissolved.

The best method was found to be:

2% pepsin in 15 c.c. .3% HCl sol.—1 hour
add 2% trypsin and .03 g. $NaHCO_3$—15 minutes
all digestion at 37°C.

CONCLUSIONS

Metacercariae may be made to excyst by artificial means. These means should follow actual physiological conditions especially in so far as pH is concerned. Results indicate that a slightly acid environment is necessary for activation to leave the last cyst wall. It may well be that the metacercariae normally emerge soon after entering the small intestine before the stomach contents are completely neutralized. The times most suited for artificial excystment seem to bear this out. (One hour in pepsin and 10-15 minutes in trypsin).

It seems that the escape from the last syst wall is due to an increased activity of the metacercaria. Presence of digestive enzymes and of products of digestion stimulate it to break through the membrane and emerge. Digestion may have some direct effect on the weak end of the cyst. The first wall, however, is simply digested away with only a trace of activity as far as the fluke is concerned. The pepsin-HCl combination dissolves away the outermost shell although some change of shape usually takes place. There is no doubt that agitation does a good deal here to shake the cyst free as well as insure optimum contact with digests.

Out of 29 metacercariae recovered, 18 were normal. In other words, 62% survived during the course of seven experiments. The response to the treatment would probably vary according to the age of the cyst. Other kinds of cysts would probably require modified treatments. These experiments on Clinostomum, however, indicate that the closer we can adhere to true physiological conditions, the more success we can expect in obtaining excysted metacercariae.

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TABLE NO. 1

	No. of Cysts	Time in Pepsin (hrs: min.)	No. Re-covered minus 1st Wall	No. in Trypsin	Time in Trypsin (min.)	Second Wall Removed	Alive
A	20	17:00	0				
B	2	1:05	2	2	0:30	1	1
C	many	2:00	many	many	0:45	1	1
D	10	1:30	8	7	0:15	3	0
E	10	1:00	2	9	0:15	1	0

1% Pepsin—Exp. D.
2% Pepsin—Exps. A, B. E
3% Pepsin—Exps. C
Alkalized with 230 mg. $NaHCO_3$
1% Trypsin—Exps. C, D
2% Trypsin—Exps. B, E

TABLE 2

	No. of Cysts	Time in Pepsin (hrs:min)	No. re-covered	1st Wall Re-moved	Time in Trypsin (min)	No. re-covered	No. Free	No. Alive
F	7	0.45	7	5	0:20	3	1	1
G	12	3:20	12	8	0:20	8	2	2
H	7	1:00	5	4	0:20	3	3	3
I	4	0:40	4	4	0:15	3	1	1
J	10	0:50	10	10	0:15	9	9	9
K	10	1:00	8	4	0:10	1	1	1
L	14	1:00	14	4	0:10	4		
M	7	0:50	4	4	0:10	1	1	1

2% solutions of Pepsin and Trypsin
Less than 50 mg. of $NaHCO_3$

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