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Effects of Pancreatin on Axenically *in Vitro* Growth of *Hymenolepis microstoma*

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Introduction

The cestode *H. microstoma* inhabting the bile duct and protruding in the duodenum; it is, therefore, under direct influence of bile and pancreatic juice. In the course of his studies with this parasite, Litchford (1963) occasionally noted flexed loop of strobila inside the pancreatic duct. We, on several occasions, have noted migration of this cestode (either completely or in parts) inside the pancreatic duct even when the dose of cysticercoids may not have produced a 'crowding effect'. From these observations, we realized that it would be interesting to know the effects of pancreatin on this cestode. There appears to be no previous report on the role of pancreatin on hymenolepidid cestodes in axenic culture.

Materials and Methods

The axenic cultivation technique used in this study was as described by Evans and DeRycke (1969) and Evans (1970). For details of this part the reader is also referred to our previous papers in these series of studies (Chowdhury and DeRycke, 1974 a, b; Chowdhury *et al.* 1974; Chowdhury and DeRycke, 1976; 1977 a, b, c).

The pancreatin used in these experiments was obtained from Merck (Germany); Art. 7131. The concentration of enzymes in this preparation is as follows:

0.1 m	Anson-E/mg Protease
0.15 U/mg	Lipase
10.0 U/mg	Amylase

The preparation was added with other ingredients (e.g. $NaHCO_3$, glucose and antibiotics) to the basal medium (BME) in four different concentrations (0.025%, 0.050%, 0.2% and 1%), and there was a control with out pancreatin added to the BME.

The basic medium consisted of three main parts: (a) Eagle's medium (=basal medium) (60%) purchased ready made from Flow Laboratories; (b) liver extract (10%) prepared from fresh lamb liver according to the method described by Sinha and Hopkins (1967); (c) serum (30%). The horse serum used in this study was obtained from Wellcome Laboratories-Lot No. Z 0279 (HS 02; No. 2).

It is to be mentioned that the results presented in the Tables 1-3; Figs. 1 and 2 and Plate 1 (Plate 1 a-e) always refer to the concentration of pancreatin in the BME. Evidently each of the *final culture media* was

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Table 1 Gross observations on the development of *H. microstoma in vitro* from 4–11 days of age in the control medium and the media containing different concentrations of pancreatin in BME (summary of three experiments)

Days post inoculation	Concentration of pancreatin per 100 ml of BME (cf. text)				
	Control (0.0%)	0.025%	0.050%	0.2%	1%
5 Pays	A =卄 P =± LPS=− GG =+	A =卅 P =± LPS=± GG =+	A =卅 P =± LPS=± GG =+	A =卅 P =± LPS=卄 GG =+	A =卅 P =± LPS=卄 GG =+
6 days	A =卄 P =± LPS=− GG =卄	A =卅 P =± LPS=+ GG =卄	A =卅 P =± LPS=+ GG =卄	A =卄 P =± LPS=卅 GG =+	A =卅 P =卄 LPS=卄 GG =卄
7 days	A =卄 P =± LPS=− GG =卄	A =卅 P =± LPS=+ GG =卅	A =卅 P =± LPS=+ GG =卅	A =卄 P =卄 LPS=+ GG =卄	A =卅 P =+ LPS=卅 GG =卅
8 days	A =卅 P =± LPS=− GG =卄	A =卅 P =+ LPS=+ GG =卅	A =卅 P =+ LPS=+ GG =卄	A =卄 P =卄 LPS=卄 GG =卄	A =卅 P =+ LPS=卄 GG =卅
9 days	A =卅 P =+ LPS=− GG =卅	A =卅 P =+ LPS=+ GG =卅	A =卅 P =+ LPS=+ GG =卅	A =卅 P =卅 LPS=卄 GG =卄	A =卄 P =+ LPS=卄 GG =卅
10 days	A =卅 P =+ LPS=− GG =卅	A =卅 P =+ LPS=+ GG =卅	A =卅 P =+ LPS=+ GG =卅	A =卅 P =卅 LPS=卄 GG =卄	A =卅 P =+ LPS=卄 GG =卅
11 days	A =卅 P =+ LPS=− GG =卅	A =卅 P =+ LPS=+ GG =卅	A =卅 P =+ LPS=+ GG =卅	A =卅 P =卅 LPS=卄 GG =卄	A =卅 P =+ LPS=卅 GG =卅

A: Activity of the worms; P: Precipitate on the tegument;
LPS: Abnormal accumulation of lipids in the proglottids and the scolex;
GG: Growth/development of gonads.
−, ±, +, , 卄, 卅, 卅 : designate intensity of above facts.

containing 60% of the concentration of the pancreatin in the BME.

During the cultivation of worms from 4–11 days of age, the media were changed on days 7, 9 and 10. The pH and osmotic pressure of each fresh and used up medium were recorded before and immediately after each experiment. The experiments were

Table 2 Effect of media containing different concentrations of pancreatin on the organogenesis of 11 days old *H. microstoma*, grown *in vitro* from 4–11 days (summary of three experiments)

No. of worms checked	Concentration of pancreatin in BME (cf. text)														
	0.0% (Control)			0.025%			0.050%			0.2%			1 %		
	Number of proglottids with			Number of proglottids with			Number of proglottids with			Number of proglottids with			Number of proglottids with		
	Sperm in CP	Ova in LS	IT	Sperm in CP	Ova in LS	IT	Sperm in CP	Ova in LS	IT	Sperm in CP	Ova in LS	IT	Sperm in CP	Ova in LS	IT
1	—	—	49	9	16	49	13	33	101	—	—	14	42	4	79
2	—	—	30	36	25	60	21	40	103	9	—	54	28	22	89
3	42	26	65	6	8	60	47	45	79	—	—	47	11	14	93
4	4	13	58	15	36	79	12	30	80	—	—	33	13	13	47
5	12	14	42	13	7	33	19	25	105	6	—	45	—	—	59
6	37	28	54	5	4	45	4	45	103	31	14	54	15	13	59
7	—	—	28	18	28	89	9	35	96	—	—	66	20	19	99
Average	13.5	11.5	46.5	14.5	17.7	59.2	17.8	36.1	95.2	6.5	2.0	44.7	18.4	12.1	70.1

CP : cirrus pouch ; LS : lateral sacs ; IT : immature testes

Table 3 Effect of media containing different concentrations of pancreatin on the quantitative distribution of calcareous corpuscles* of 11 days old *H. microstoma*, grown *in vitro* from 4–11 days

Serial number of proglottids**	Concentration of pancreatin per 100 ml of BME (cf. text)				
	0.0% (Control)	0.25%	0.050%	0.2%	1 %
1	13.5	20.0	25.7	15.4	25.5
2	21.1	24.5	29.2	17.5	31.8
3	25.8	22.4	31.1	18.7	32.4
4	29.8	31.4	27.7	18.5	34.2
5	30.7	24.7	33.2	18.1	29.4
6	33.1	30.0	36.8	17.5	23.7
7	28.0	30.7	45.5	20.8	29.4
8	32.8	29.8	43.7	21.1	29.1
9	34.5	35.2	35.0	19.8	29.0
10	32.0	32.4	38.7	20.8	26.8
Totals	281.2	281.2	346.6	188.2	291.3

Each figure is the average of determinations on 7 different worms.

* Calcareous corpuscles present in the last ten proglottids excluding the end-proglottid. Only those corpuscles measuring 15–20 μm in size (cf. Chowdhury and DeRycke, 1974 a) have been counted.

** The second proglottid (next to the end-proglottid) represents the firist proglottid.

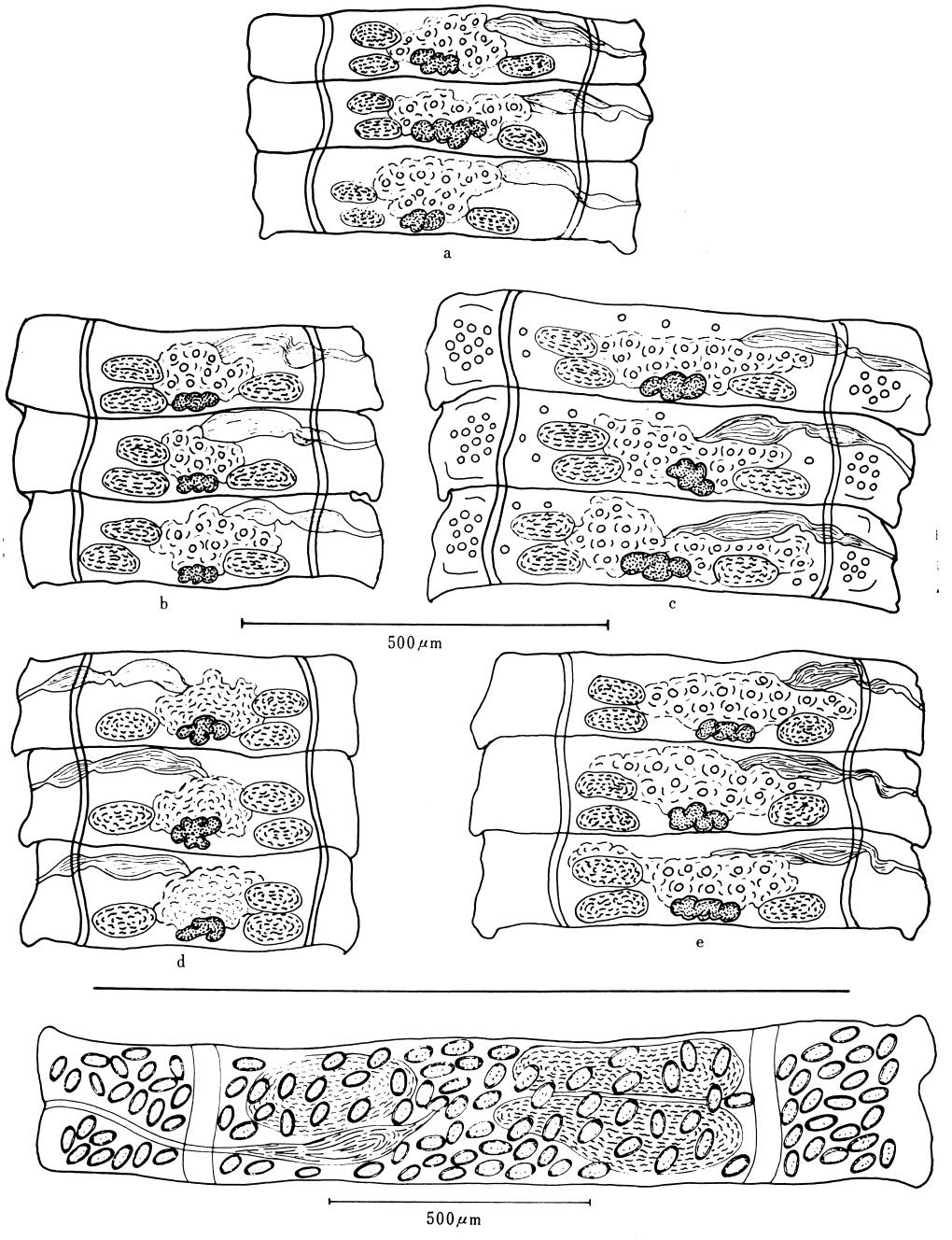

Plate 1 a-e : Illustration of organogenesis in 11-day-old *Hymenolepis microstoma*, grown *in vitro* from 4-11 days (cf. Table 2). The figure in the bottom is from an 11-day-old *in vivo* grown worm for comparison (all figures are drawn from nearly the same region of the worm) :

a Proglottids of a worm grown in the medium without pancreatin in BME.
b Proglottidis of a worm grown in the medium containing 0.025% pancreatin in BME.
c Proglottids of a worm grown in the medium containing 0.050% pancreatin in BME Note the 'lateral sacs' containing fertilized ova.
d Proglottids of a worm grown in the medium containing 0.2% pancreatin in BME. Note the retardation in oogenesis.
e Proglottids of a worm grown in the medium containing 1 % pancreatin in BME. Compare the ovary with other in a, b, c and d.

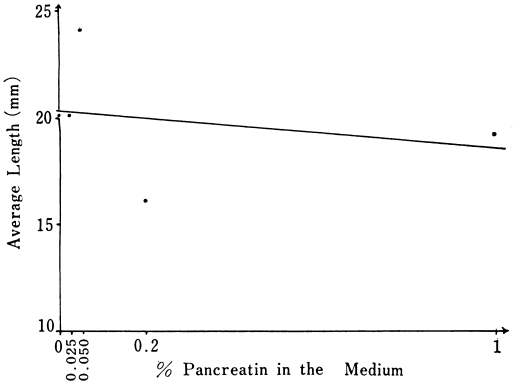

Fig. 1 Showing the average length of 11 days old worms (n=20) grown in the media containing different concentrations (0.025%, 0.050%, 0.2% and 1%) of pancreatin in BME and in the control medium without pancreatin in BME (regression line : y= 20.4-1.9x ; $P>0.05$).

repeated thrice. From the total of 45 cultured worms from each medium, 20 largest worms were selected for measuring the length (Fig. 1) and other studies. The length was measured on day 11, keeping the worms in Ringer's (37.5C) for 1 h at room temperature (±25C) after a brief rinsing in Hanks' solution (37.5C). This procedure was found necessary to prevent shrinkage and frequent rupture of the tegument of 'weak' *in vitro* grown worms.

Beside the general growth, length, pH and osmotic pressure of different media, the other criteria used for evaluation of worms, were organogenesis, quantitative distribution of calcareous corpuscles, qualitative distribution of neutral fats and phospholipids in the parenchyma or other tissues of worms. The detailed procedures adopted in these studies, have been described in the relevant papers mentioned above.

Results

The observations on different experiments during development of *H. microstoma in vitro* from 4-11 days of age have been summarized in Tables 1-3 ; Plate 1 a-e ; Figs. 1 and 2. Although it has been observed that growth of worms varies with increasing concentration of pancreation in BME, there is, however, a considerable inhibition in development at 0.2 % concentration. The spermatogenesis and oogenesis in worms grown in this concentration are found lagging behind even that of the control medium (Fig. 1 and Table 2). From the Table 2 and Fig. 1, it could further be concluded that the best development has been achieved in the medium containing 0.050 % pancreatin in BME.

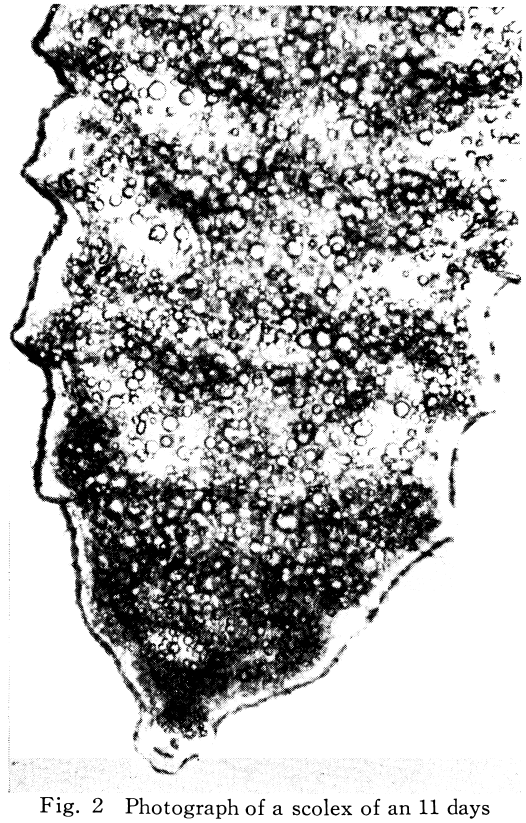

Fig. 2 Photograph of a scolex of an 11 days old worm grown in the medium containing 1 % pancreatin in BME (coverslip preparation). Note the accumulation of fat droplets on and around the suckers (× 140).

From the illustrations, it is, however, imperative that the cultured worms from all concentrations of pancreatin, including those from the controls, are retarded in growth in comparison to the *in vivo* grown worms of the same age (compare Plate 1 a-e

Fig. 3 Posterior third of an 11 days old worm grown *in vitro* in the medium containing 1% pancreatin in BME (coverslip preparation). Observe the heavy concentration of fat globules in the parenchyma (× 140).

with the figure in the bottom in Plate 1).

The accumulation of excess neutral fats appears to be maximum in the worms grown in the medium containing 1% and 0.2% pancreatin in BME. By this accumulation of lipids in the parenchyma, the latter looks dark (Fig. 3). The excess concentration of lipids in the scolex is even differentiable under the inverted microscope. The major sites of accumulation of fat globules in the scolex are on the suckers (Fig. 2), on and around the rostellum, and in the neck region. With the decreasing concentration of pancreatin in the BME, the excess deposition of lipids, however, disappears. The phospholipids are found in the proximal and distal cytoplasm, reproductive organs, nerve fibres and carcareous corpuscles.

From the results presented in the Table 3, there appears to be some effect of pancreatin on the distribution of calcareous corpuscles at a concentration of 0.050% in the BME.

Changes in the pH of different media—although somewhat different from their osmotic pressure—do not show much variability (Fig. 4).

Discussion

Earlier studies have shown that cestodes are capable of modifying the effects of host's digestive enzymes, particularly those of pancreatic origin (Taylor and Thomas, 1968; Reichenbach-Klinke and Reichenbach-Klinke, 1970; Reads, 1973; Pappas and Read, 1972 a, b; Ruff and Read, 1973).

Taylor and Thomas (1968) have observed that in the presence of *H. diminuta*, *H. microstoma* and *Moniezia expansa*, hydrolysis of starch by a bacterial α-amylase was enhanced and that the enhancement was related to the surface area of the worms. Read (1973) has also shown that in the presence of *H. diminuta* the activity of the pancreatic α-amylase is increased (although intact worms did not show amylotytic activity themselves). He further clarified that amylase activity is: (1) proportional to the number of worms present in the substrates, (2) that the relative enhancement occurs at a very low enzyme concentrations, (3) that the amylase activity is readily reversible by polycations and (4) not exhibited in the presence of dead worms.

On the contrary, Ruff and Read (1973) have demonstrated that the activity of pancreatic lipase is inhibited in the presence of *H. diminuta*. The inhibition is: (1) constant over an enzyme concentration ranging from 0.4–2.0 U/ml, (2) dependent upon the pH, since at higher pH an enhanced activity was observed, (3) reversible by removing the worms from the enzyme and, (4) not blocked by polyions. The advantage of lipase inhibition to *H. diminuta*, however, could not be explained.

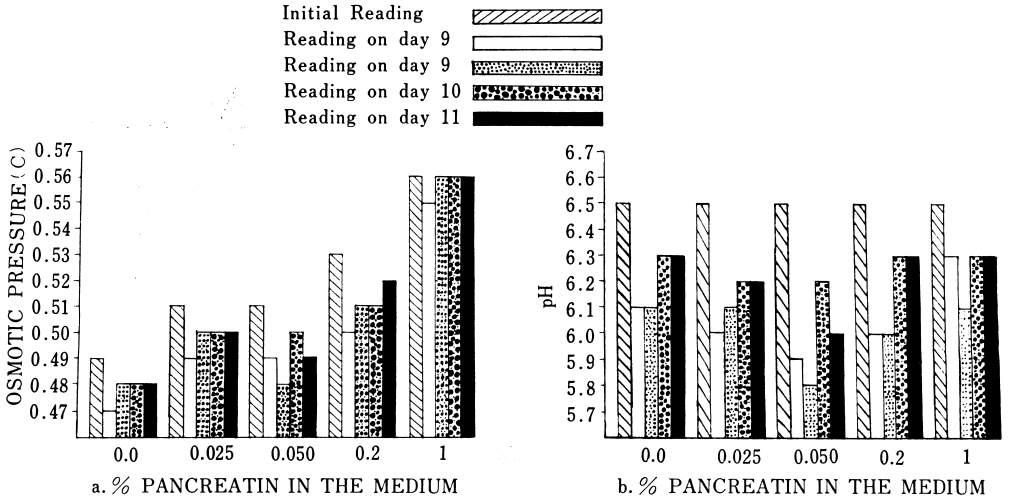

Fig. 4 Bar diagrams showing changes in osmotic pressure (a) and (b), pH respectively of different media during *in vitro* cultivation of *H. microstoma* from 4 day to 11 day, containing different concentrations of pancreatin in BME and in the control medium without pancreatin in BME.

With regard to the pancreatic proteolytic enzymes, Pappas and Read (1972 a, b) have shown that trypsin is reversibly inactivated in the presence of *H. diminuta* and a similar effect is also noted in respect of α- and β-chymotrypsin.

Several simple organic compunds are also known to act as inhibitors of proteolytic enzymes, e.g. organophosphates. Trypsin inhibitors of protein nature are widely distributed in the nature and have also been isolated in crystalline form from a variety of substances including bovine serum (Gray *et al.*, 1960). In higher animals it is known that digestive enzymes are more active in presence of bile salts and that bile salts are also present in the serum (Haslewood, 1967).

From the results presented in the Table 2 it can be observed that at one of the two lower concentrations of pancreatin (i.e. at 0.050% pancreatin in the BME) worms show better organogenesis than in the controls. By increasing the concentration of pancreatin four times (i.e. 0.2% pancreatin in the BME) worms show inhibition in development at the same time organogenesis in them is effected which is evident when compared with the control worms (Table 2; Fig. 1). The inhibition of growth is decreased by increasing further the concentration of pancreatin to 1% (i.e. five times) in the BME. The groath and organogenesis, however, remain nearly the same as the controls. From the Table 2, it is further evident that the most suitable concentration in which worms show best growth and organogenesis is 0.050% pancreatin (in BME). In this concentration worms have: (1) highest number of progrottids with immature testes and (b) maximum proglottids containing fertilized ova in the 'lateral sacs'.

Bailey and Fairbairn (1968) and Frayha and Fairbairn (1968) have shown that in the presence of bile salts there is an increased accumulation of lipids in *H. diminuta*. Our previous experiments with ox gall (=dehydrated bile) have shown similar results with *H. microstoma* (Chowdhury *et al.*, 1974). The observations of the present experiments also have indicated that pancreatin causes accumulation of lipids and that, accumulation of neutral fats in abnormal locations increases with the increasing concentration of pancreatin (in BME). The possible significance of phospholipids in cestodes has been discussed in a relevant paper by us (Chowdhury and DeRycke, 1976).

From the Table 3, it is obvious that there is an increase in the quantitative distribution of calcareous corpuscles in the proglottids of worms grown in the medium containing 0.050% pancreatin (in BME). This appears to be related to the better general condition of the worms in this medium.

If the various results of the present experiments are critically analysed, the existence of both stimulatory and inhibitory effects of added pancreatin to the culture medium can not be ruled out. The effects are thought to be related enzymatically to other factor(s) present in the complex medium.

Summary

H. microstoma has been cultured *in vitro* from 4 to 11 days of age, using Eagle's medium supplemented with horse serum, lamb liver extract and pancreatin in four different concentrations (0.025%, 0.050%, 0.2% and 1% all in BME) including a control without pancreatin. The results of the experiments have indicated that growth is not much stimulated by increasing the concentration of pancreatin. By adding 0.2% pancreatin (in BME) organogenesis is slowed down but can be improved slightly by increasing the concentration to 1% (in BME). The results of the latter concentration become nearly parallel with the controls.

In these experiments worms show best results particularly with respect to oogenesis and distribution of calcareous corpuscles at a concentration of 0.050% pancreatin (in BME). In the highest two concentrations, neutral lipids accumulate in excess in the parenchyma of the proglottids, the suckers, in and around the rostellum and in the neck region.

From the results of various experiments it is concluded that pancreatin: (a) causes an increased accumulation of neutral lipids, (b) may stimulate oogenesis at appropriate concentrations, (c) inhibits growth only at certain concentration, possibly due to interactions of the enzymes with the other factor(s) present in the medium.

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Hymenolepis microstoma の生体外発育におよぼすパンクレアチンの影響

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H. microstoma を Eagle 液に馬血清, 子羊肝エクストラクトおよび4段階の濃度 (0.025 %, 0.05 %, 0.2 % および1 %) のパンクレアチン溶液中で, 4日令から11日令まで培養した. 対照にはパンクレアチンを含まないものを用いた. パンクレアチンの濃度を高めても虫体の成虫がいちじるしく増大するということはなく, 0.2 %パンクレアチン濃度では器官形成が遅延したが, それを1 %にするとやや改善され, ほぼ対照と同程度となつた.

卵子形成と石灰顆粒の分布に関しては, 0.05 %パンクレアチン濃度で最良の成績が得られた. 0.2および1 %では中性脂肪が体節, 吸盤, 吻, 頸部の柔組織に大量に充積した.

本実験の結果から, パンクレアチンは (1) 中性脂肪の増大と充塡を来たし, (2) 適量ならば卵子形成を刺激し, (3) ある濃度では成長を抑制する, と結論された.