Studies on *Hymenolepis microstoma* in vitro

I. Effect of Heme Compounds on Growth and Reproduction

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**Summary.** The effect of various heme compounds on growth and maturation of *Hymenolepis microstoma* in vitro has been determined. At 4 days p.i., worms were collected and cultured in vitro for 6 days in a medium containing various concentrations of haemoglobin, hemin, and bilirubin.

Addition of haemoglobin to a medium resulted in significant increases in length, wet weight, number of immature and mature proglottids as compared to the control medium. Supplements of hemin did not seem to affect worm length; however, they caused a significant weight increase, and the numbers of immature and mature proglottids were also more than those in control medium. Bilirubin supplements failed to show any effect on growth and maturation of *H. microstoma*, and the experimental and control worms were found similar in all respects.

In the light of the success with haemoglobin and hemin as growth promoting substances, the role of blood ingredients was also considered. For strobilization and maturation of *H. microstoma* in vitro, the presence of some kind of heme protein seems essential.

**Introduction**

Evans (1970) reported the first successful culture of *Hymenolepis microstoma* from cysticercoid to egg producing adult. We tried to repeat his procedures in this laboratory but failed repeatedly, probably due to lack of specific horse serum (Khan and De Rycke, 1975). In various sera utilized, the excysted worms remained alive and normal for many days but no strobilization was achieved. Seidel (1971) showed that *H. microstoma* could be grown from the juvenile to prepatent adulthood in a diphasic culture system. The worms achieved strobilization and maturation only when either hemin or blood was present in the medium.

The present studies were undertaken as to find out how heme compounds would act on growth and gametogenesis in already strobilated worms.

**Materials and Methods**

NMRI male mice of 6–7 weeks old were infected with 50 cysticercoids each, and after 4 days young worms were collected in sterile conditions by the following method: mice were killed by a heavy dose of ether, a portion of liver with intact bile duct and about 10 cm of small intestine were removed and washed in sterile Hanks' solution (containing 550 mg glucose per 100 ml, with neutral pH; at 37°C). The intestine was slit open from the ileocecal point towards the stomach while taking care not to cut the worms. The bile duct

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was left intact because at this age, i.e. 4 days p.i., the worms were quite small (about 2.5 to 3.5 mm in length) with most of the body still inside the bile duct, and due to the large infection dose, there was a high probability that some of them might be cut or damaged. About 4-5 opened intestines were placed over a sieve and incubated for 1/2 h in sterile Hank's solution at 37°C. The undamaged worms so recovered were washed three times in Hank's solution and care was taken to inoculate 5 worms of equal length in preincubated culture tubes, in duplicate. These tubes were placed over a roller drum at an angle of 23°. Each culture tube (16 × 120 mm) contained 6 ml of culture medium and the gas phase was 5% CO₂ in N₂. In all experiments medium M 6/9 (cf. Khan and De Rycke, 1975) was used, serum and liver extract being from the same batch, respectively.

After specified days the cultures were stopped and the worms were washed in Hank's solution. For length measurements they were immediately transferred to numbered petri dishes containing distilled water, and kept in the refrigerator at 4°C for 10 min. In this way 4–5 relaxed worms were transferred to a glass slide and after recording the length the excess of water was withdrawn with the help of a Pasteur pipette and 10% formalin was introduced. The worms were pressed lightly with a cover glass and the slide was put in a large covered petri dish containing a little formalin. The worm samples used for the length measurements were utilized later on for the study of developmental morphology and proglottid counts. The whole organisms were stained with Borax Carmine, counting and differentiation of proglottids was based on the following procedure: counting of all the proglottids was begun from the last proglottid of the posterior end and continued towards the scolex. Only those proglottids were considered mature which had spermatozoa in the seminal vesicle and cirrus pouch. The proglottids which showed the reproductive organs in the process of formation but were not yet reproductively functional (absence of spermatozoa) were regarded as immature. The proglottids immediately behind the scolex were excluded from the counting if they were devoid of genital primordia.

Fresh weight of 10 day old worms was determined immediately after stopping the culture. The worms were washed in Hank's solution and weighed in foil cups after removing excess moisture with filter paper.

Haemoglobin was obtained from Merck, Germany, and dissolved in BME solution in required quantities. The final concentration of haemoglobin (Hb) in the culture medium varied between 0.05% and 1%.

Hemin (equine) was obtained from Sigma, U.S.A., and the stock solution was prepared as described by Seidel (1971). The concentration in the medium varied from 0.05% to 0.3%.

Bilirubin was procured from Merck, Germany, and the final concentration in the medium was adjusted between 0.0005% and 0.01%.

Results

Effect of Haemoglobin on Growth and Reproduction of 4–10 Day Old H. microstoma

To determine the effect of haemoglobin on the development of H. microstoma, the worms were incubated in four different concentrations of the supplement (cf. Table 1). The experimentally grown worms (in Hb supplemented media) were compared against controls (without Hb) for length, immature, mature and total numbers of proglottids on days 6, 8 and 10. Comparative studies of weight measurements were carried out on 10 day old worms only. The average length of 4 day old in vivo worms was 3.0 mm (n > 80).

In the higher concentrations of the haemoglobin supplement, i.e. 1% and 0.5%, worms were retarded in growth. The worms in these media were sluggish and slow in their movements; however, visually they were clean and without any apparent blemish. In Table 1, it can be seen that growth in 0.1% and 0.05% Hb was very encouraging. Statistically, with 0.1% Hb supplemented medium the worms were significantly larger (P < 0.05) than controls on either day 8 or day 10.