[Jap. J. Parasit., Vol. 28, No. 5, 273-284, 1979]

Comparative Studies on the Development of *Hymenolepis microstoma in Vitro* in Different Types of Sera

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(Received for publication; August 6, 1978)

Introduction

The cultivation of helminths in general is a very complex challenge and presents many difficult problems. These hindrances often arise from the lack of a suitable serum which should support reasonable growth of the parasite *in vitro*.

During the cultivation of *H. microstoma* from the cxcysted stages, at the outset, it was noted that young adults survived in the medium containing a specific horse or calf serum for a maximum period of 4-5 days before being disintegrated completely in it.

Though it is well recognised that serum constitutes a vital part of the culture media for most helminths, many of its effects on the development of the parasite are unknown. The observations of our preliminary experiments led to a detailed study of the effects of different calf or horse sera on general growth, development and organogenesis of *H. microstoma*, from day 4 to day 11 on a histological and histochemical basis.

In recent years considerable progress has been made in culturing larval stges of cestodes *in vitro*. However, the criteria used by us in evaluating axenically grown *H. microstoma* appear to be new and interesting especially in a hymenolepidid cestode commonly employed for laboratory experimentations.

Materials and Methods

The source and way of maintaing the host and parasitic material and related experimental procedures adopted were the same as described earlier by us (Chowdhury and DeRycke, 1976 a, b).

The axenic *in vitro* cultivation method used was as described by Evans and De Rycke (1969) and Evans (1970).

The culture medium consisted of main three parts: (a) Eagle's medium (BME) (60%) purchased ready made from Flow Laboratories; (b) liver extracct (10%) prepared from the fresh lamb liver according to the method described by Sinha and Hopkins (1967); (c) calf or horse serum (30%). The Eagle's medium contained 0.1 ml of a streptopenecillin and 0.1 ml of fungizone (Squibb) preparations/100 ml (cf. Chowdhury and De Rycke, 1977).

A total of seven sera were selected as components for the different media. Of these, two were calf sera obtained locally (L) and five were horse sera of which one was from Flow Laboratories (F) and the other four from Burroughs Wellcome (W). All the sera, if otherwise not done by the concerned firms, were heated in the labora-

An abstract of this paper has been presented at the First National (Indian) Parasitological Congress, February 24-26, Baroda, 1977

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tory to 56 C for half an hour before being used in the experiments. Due to variability in the effects of different sera the source and batch number of each serum has been referred at appropriate places.

The 4 day young adults used in the experiments were recovered in a crystalline dish over plastic sieve. Before transferring them in groups of 5 to roller tubes the young adults were carefully washed in Hanks' solution (containing antibiotics. at least 4 times so as to remove the digestive enzymes or attached debris. Each roller tube contained 5 ml of culture medium which has been gassed for 30 seconds with a mixture of 95% N_2+5% CO_2, and had been incubating at 3.75 C for about 2-3 h. After receiving the worms, the tubes were re-gassed for another 30 seconds and then placed in a roller drum in the incubator at 37.5 C. The medium was changed on days 7,9 and 10. The pH and osmotic pressure of fresh and used up medium in each tube were recorded before and after each experiment. Each determination of osmotic pressure is the average of 3 readings (same tube) and each figure (Fig. 7b) is the average of 5 determinations (different tubes).

Gross observations were made from coverslip preparations in Ringer's. The illustrations were photomicrographed from fresh specimen or drawn *in situ* from permanent whole mounts (borax carmine stained) with the aid of a camera lucida. The detailed procedures of the evaluation of the criteria of the cultured worms in each medium, histological and histochemical methods employed have been described in the relevant papers by us (see Chowdhury and De Rycke, 1974 a, b, 1976 a, b, 1977).

Results

Gross observations during the *in vitro* development of *H. microstoma* from day 4 to day 11 in media with different types of sera have been summarised in Tables 1, 2 and 3. From the results it will be evident that there is a marked difference in the activity, growth and development of worms in the media containing dffferent sera. Also the formation of precipitate on the tegument, quantitative distribution of calcareous corpuscles and accumulation of neutral fats in the parenchyma of worms vary from serum to serum.

The sequence of organogenesis in 11 day old worms in different sera have been detaiiled in Table 3 and illustrated further in Plate 1 (a to g). From the illustrations it can be inferred that the *in vitro* development of the worms is lagging behind as compared to *in vivo* (compare Plate. 1 a to g with bigure in the bottom). Further gametogenesis is slower in worms grown in the calf sera than in the horse sera (compare Plate. 1 a and b with d to g). In some horse sera spermatogenesis and oogenesis appear to vary considerably (compare Plate 1 c with g).

There is a marked variation in the distribution of calcareous corpuscles in each group of worms but there appears to be a parallelism between the numbers of proglottidal calcareous corpuscles observed and general growth and organognesis of the parasite *in vitro* (cf. Tables 2, 3 and 4).

In fact, both histologically and histochemically, it has been observed that worms grown in most of the media with horse sera contain appreciable amounts of neutral lipids (Fig. 6) comparable to 7-9 day old *in vivo* grown worms. On the contrary, the worms grown in the media with calf sera either contain very less or do not contain neutral fats in their parenchyma (see Figs. 4, 5, Table 1). Although neutral lipids vary, no difference was observed in the qualitative distribution of phospholipids in young adults grown in media containing either horse or calf serum as compared to worms developed in the definitive host.

In these experiments the maximum change in pH was a decrease of 1.1 after 7 days of culture (day 4 to day 11); the maximum change in Δ was a decrease of 0.03 also after 7 days of culture (Figs. 7 a, b).

Table 1 Gross observations on the development of *H. microstoma in vitro* from days 4–11 of age in media with different sera* (Summary of three experiments)

Days post inoculation	Code number of sera used						
	CS-1(L) Lot No. XVIII	CS-2(L) Lot No. XIX	HS-1(F) Lot No. L 40272	HS-2(W) Lot No. K 4062	HS-3(W) Lot No. Z 0270	HS-4(W) Lot No. Z 0218	HS-5(W) Lot No. Z 0239
5 days	A =卄	A =卄	A =卅	A =卅	A =卄	A =卄	A =卅
	P =−	P =−	P =−	P =+	P =±	P =−	P =+
	LP=±	LP=+	LP=+	LP=卄	LP=卄	LP=卄	LP=卄
	GG=−	GG=+	GG=卄	GG=卅	GG=+	GG=+	GG=+
6 days	A =卄	A =卄	A =卅	A =卅	A =卄	A =卅	A =卅
	P =−	P =±	P =+	P =卄	P =±	P =±	P =+
	LP=−	LP=±	LP=+	LP=卄	LP=卄	LP=卄	LP=卄
	GG=±	GG=+	GG=卄	GG=卅	GG=卄	GG=卅	GG=卅
7 days	A =卅	A =卅	A =卅	A =卅	A =卄	A =卅	A =卅
	P =−	P =±	P =卄	P =卄	P =±	P =±	P =+
	LP=−	LP=−	LP=+	LP=卄	LP=卄	LP=卄	LP=卄
	GG=+	GG=+	GG=卄	GG=卅	GG=卄	GG=卅	GG=卅
8 days	A =卅	A =卅	A =卅	A =卅	A =卅	A =卅	A =卅
	P =−	P =+	P =卄	P =卄	P =±	P =±	P =±
	LP=−	LP=−	LP=+	LP=卅	LP=卄	LP=卄	LP=卄
	GG=+	GG=+	GG=卄	GG=卅	GG=卄	GG=卅	GG=卅
9 days	A =卅	A =卅	A =卅	A =卅	A =卅	A =卅	A =卅
	P =−	P =+	P =卄	P =卄	P =+	P =±	P =−
	LP=−	LP=−	LP=+	LP=卅	LP=卅	LP=卄	LP=卅
	GG=+	GG=+	GG=卄	GG=卅	GG=卅	GG=卅	GG=卅
10 days	A =卄	A =卅	A =卅	A =卅	A =卅	A =卅	A =卅
	P =−	P =+	P =卄	P =卄	P =+	P =±	P =−
	LP=−	LP=−	LP=+	LP=卅	LP=卅	LP=卄	LP=卅
	GG=+	GG=+	GG=卄	GG=卅	GG=卅	GG=卅	GG=卅
11 days	A =卄	A =卅	A =卅	A =卅	A =卄	A =卅	A =卅
	P =−	P =+	P =卅	P =+	P =+	P =±	P =−
	LP=−	LP=−	LP=+	LP=卅	LP=卅	LP=卅	LP=卅
	GG=+	GG=+	GG=卄	GG=卅	GG=卅	GG=卅	GG=卅

* See also Table 2, 3 and 4

A=Activitiy of worms in the medium

P=Precipitate on the tegument

LP=Normal accumulation of lipids in the proglottids

GG=Growth and development of gonads

−=retarded(GG)/absent or none(A; P; LP)

±=very less (P; LP; GG)

+=little (P; LP; GG)

卄=moderate (A; P; LP)/good (GG)

卅=moderate to much (A; P; LP)/good to very good (GG)

卅=vigorous (A)/very good (GG)

Table 2 Effect of media with different sera on the length of 11-day-old *H. microstoma*, grown *in vitro* from 4-day-old young adults (cf. Table 3)

Code number of sera used	Average length in mm (n=20)	Standard error
CS-1 (L) Lot No. XVIII	10.40	0.563
CS-2 (L) Lot No. XIX	9.50	0.498
HS-1 (F) Lot No. L 40272	11.00	0.579
HS-2 (W) Lot No. K 4062	17.25	1.144
HS-3 (W) Lot No. Z 0279	20.04	0.514
HS-4 (W) Lot No. Z 0218	26.95	1.308
HS-5 Lot No. Z 0239	35.64	1.706

Discussion

Pioneering works of Healy *et al.* (1955) and subsequently of Tozer and Pirt (1964), Healy and Parker (1966), Schaer and Schindler (1976), Shodell and Rubin (1970) have shown that either whole and fractioned animal blood serum is essential in tissue culture techniques. Wallis *et al.* (1969) suggested that serum probably stimulates RNA and DNA synthesis (and consequently mitosis) since its absence cell growth ceases.

Using either rabbit or sheep serum Heath and Smyth (1970) described the *in vitro* growth of *Echinococcus granulosus*, *Taenia pisiformis*, *T. serialis*, *T. hydatigera* and *T. ovis*. Heath and Elsdon-Dew (1972) again reported the *in vitro* development of *T. saginata* and *T. taeniaeformis* larvae in calf, sheep, rat and rabbit sera. Heath and Smyth (1970) and Heath and Elsdon-Dew (1972) have pointed out that the different sera they used in their studies did not support growth of larvae equally and that a specific serum was a must for successful *in vitro* development of each parasite. However, they also felt that no generalisation could be made with regard to the suitability of a serum.

As has been mentioned earlier all horse sera, although better than calf sera, did not support growth of *H. microstoma* equally. While dealing with large numbers of calf or horse sera throughout all our studies, it was found that in one occasion a recalcified and oxalated horse serum (HS-22, Lot No. K 4358) from Wellcome Laboratories) stimulated growth from the excysted stages of this cestode to the egg bearing adults in 18 days. From the results presented in the Tables 1, 2 and 3 it could be observed that there is a marked inhibition of growth and organogenesis in the calf sera. The same is the case with most of the calf sera used throughout our experimental period. Diamond (1970) observed a similar variability in growth of *Entamoeba histolytica* in bovines rabbit, lamb and human sera he used. In the cultivation of *Trichinella spiralis* larvae several workers have reported effects of different sera to varying degrees (Weller, 1943 ; Kim, 1961 ; Meerovitch, 1962 ; Tarkanov, 1964).

The effects of different sera on parasites have been variously explained. Thus Heath and Elsdon-Dew (1972) found that some sera taken from old rats had a stimulating effect on growth of *T. taeniaeformis* larvae while serum from older calves inhibited growth of *T. saginata* larvae. From these results they concluded that substances which inhibited growth of the *T. saginata* larvae may be formed during the foetal stage or that factors which could stimulate growth are absent in this type of serum. By detecting large amounts of globulins in the foetal calf sera these workers later suggested that the globulins may be associated either with stimulation or inhibition of growth.

Terry (1957) while working with *Trypanosoma vivax* reported the presence of a trypanosomacidal factor in the β-globulin range of the cotton rat serum ; on the contrary, Greenblatt *et al.* (1969) found a stimulatory factor for *Trypanosoma lewisi* in the same range. Wallis *et al.* (1969) found the presence of an antiprotease (=antienzyme)

Table 3 Effect of media with different sera on the organogenesis of 11-day-old *H. microstoma* grown *in vitro* from 4-day-old young adults

No. of worms checked	Code number of sera used																							
	CS-1 (L) Lot No. XVIII			CS-2 (L) Lot No. XIX			HS-1 (F) Lot No. L-40272 Cat No. 4010D			HS-2 (W) HS 52 Lot No. K4026			HS-3 (W) HS 02 Lot No. Z0279			HS (W) HS 02 Lot No. Z0218			HS-5 (W) HS 02 Lot No. Z0239		
	Number of proglottids with			Number of proglottids with			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	IM	Sperm in CP	Ova in LS	IM	Sperm in CP	Ova in LS	IM	Sperm in CP	Ova in LS	IM	Sperm in CP	Ova in LS	IM	Sperm in CP	Ova in LS	IM	Sperm in CP	Ova in LS	IM
1	—	—	32	—	—	36	9	—	50	50	29	52	42	26	65	72	30	149	90	70	132
2	—	—	51	—	—	38	—	—	45	75	21	40	37	28	54	56	28	92	47	25	83
3	—	—	28	—	—	27	—	—	34	—	—	51	12	8	54	34	21	85	125	55	194
4	—	—	12	—	—	33	—	—	61	—	—	24	4	13	58	48	11	105	48	32	110
5	—	—	24	—	—	27	—	—	70	58	14	54	18	14	63	27	27	49	78	34	123
6	—	—	10	—	—	16	—	—	42	14	3	58	31	20	49	100	27	153	100	57	152
7	—	—	18	—	—	24	5	4	44	—	—	44	12	14	42	72	13	93	72	48	117
8	—	—	22	—	—	22	—	—	30	50	8	54	—	—	30	113	23	141	56	39	121
9	—	—	15	—	—	27	—	—	28	—	—	36	—	—	49	80	25	112	79	54	131
10	—	—	16	—	—	27	—	—	68	—	—	42	—	—	28	52	32	70	94	73	129
Average	—	—	22.8	—	—	27.7	1.4	0.4	46.8	24.7	7.5	45.5	15.6	12.3	49.2	65.4	23.7	104.9	78.9	48.7	129.2

CP=Cirrus Pouch
LS=Lateral Sacr
IM=Immature testes

Table 4 Effect of media with different sera on the quantitative distribution of calcareous corpuscles* of 11-day-old *H. microstoma*, grown *in vitro* from 4-day-old young adults

Serial number of proglottid**	Code number of sera used						
	CS-1 (L) Lot No. XVIII	CS-2 (L) Lot No. XIX	HS-1 (F) Cat. No. 4-010D L 40272	HS-2 (W) HS 52 Lot No. K4062	HS-3 (W) HS 02 Lot No. Z0279	HS-4 (W) HS 02 Lot No. Z 0218	HS-5 (W) HS 02 Lot No. Z0239
1	1.8	1.2	10.1	18.4	13.5	47.5	48.1
2	3.4	1.5	11.1	28.4	21.1	45.5	69.8
3	1.7	2.2	12.7	26.0	25.8	51.2	65.5
4	2.7	3.4	16.0	33.5	29.8	56.0	78.1
5	2.5	4.2	14.0	31.2	30.7	54.4	78.2
6	3.4	4.4	17.2	30.8	33.1	59.2	75.8
7	2.7	3.8	15.7	32.8	28.0	60.0	81.2
8	3.2	4.0	17.4	30.8	32.7	50.8	72.2
9	3.4	5.0	16.2	28.7	34.5	57.0	79.4
10	5.1	4.1	17.2	27.7	32.0	57.5	83.0
Totals	29.9	33.8	109.0	288.3	281.2	539.1	731.3

* Calcareous corpuscles present in the last ten proglottids excluding the end-proglottid. Only those concretions measuring 15–20 μm in size (cf. Chowdhury and De Rycke, 1974 a) have been counted.
** The second proglottid (next to the end-proglottid) represents the first proglottid.
Each figure is the average of determinations on seven different worms.

factor in large numbers of animal and human sera. This anti-enzyme only produced by α-and β-globulins, has an effect on adverse (harmful) enzymes secreted by developing cell monolayers and is apparently needed for successful tissue culture. These workers further suggested that those sera which do not support growth of tissue cultures lack this anti-enzyme factor.

The inhibitory effects produced by certain

Plate 1 a–g : Illustrations of organogenesis in 11-day-old *Hymenolepis microstoma*, grown *in vitro* from 4–11 days (cf. Table 3). The figure in the bottom is from an 11-day-old *in vivo* grown worm forcomparison (all figures are drawn nearly from the same region of the worm):

a Proglottids of a worm grown in the medium containing calf serum CS-1 (Lot No. XVIII).

b Proglottids of a worm grown in the medium containing calf serum CS-2 (Lot No. XIX). Note the poorly developed testes; also observe the underdevloped female reproductive glands.

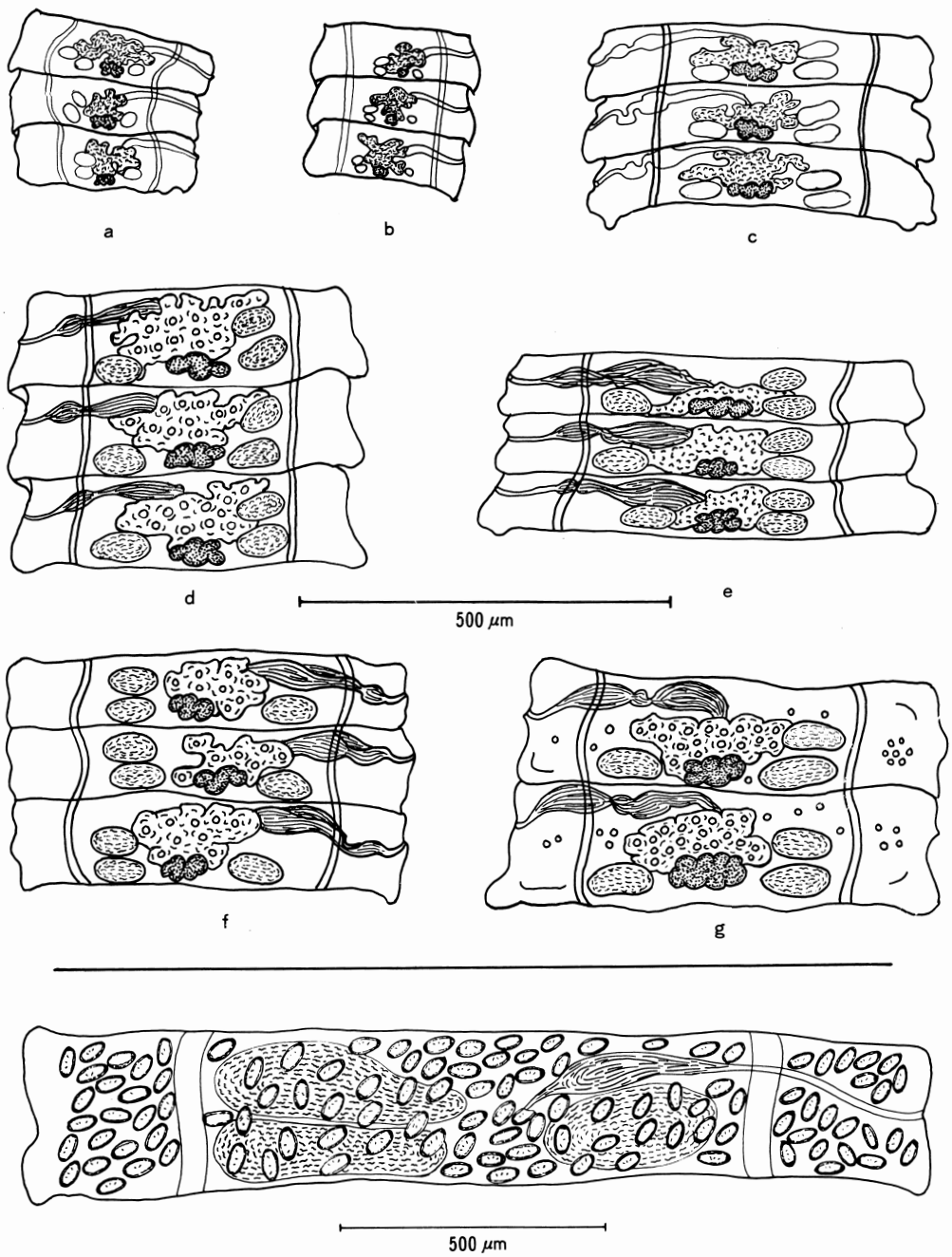
c Proglottids of a worm grown in the medium containing horse serum HS-1 (Lot No. L 40272).

d Proglottids of a worm grown in the medium containing horse serum HS-2 (Lot No. K 4062).

e Proglottids of a worm grown in the medium containing horse serum HS-3 (Lot No. Z 0279).

f Proglottids of a worm grown in the medium containing horse serum HS-4 (Lot No. Z 0218).

g Proglottids of a worm grown in the medium conraining horse serum HS-5 (Lot No. Z 0239).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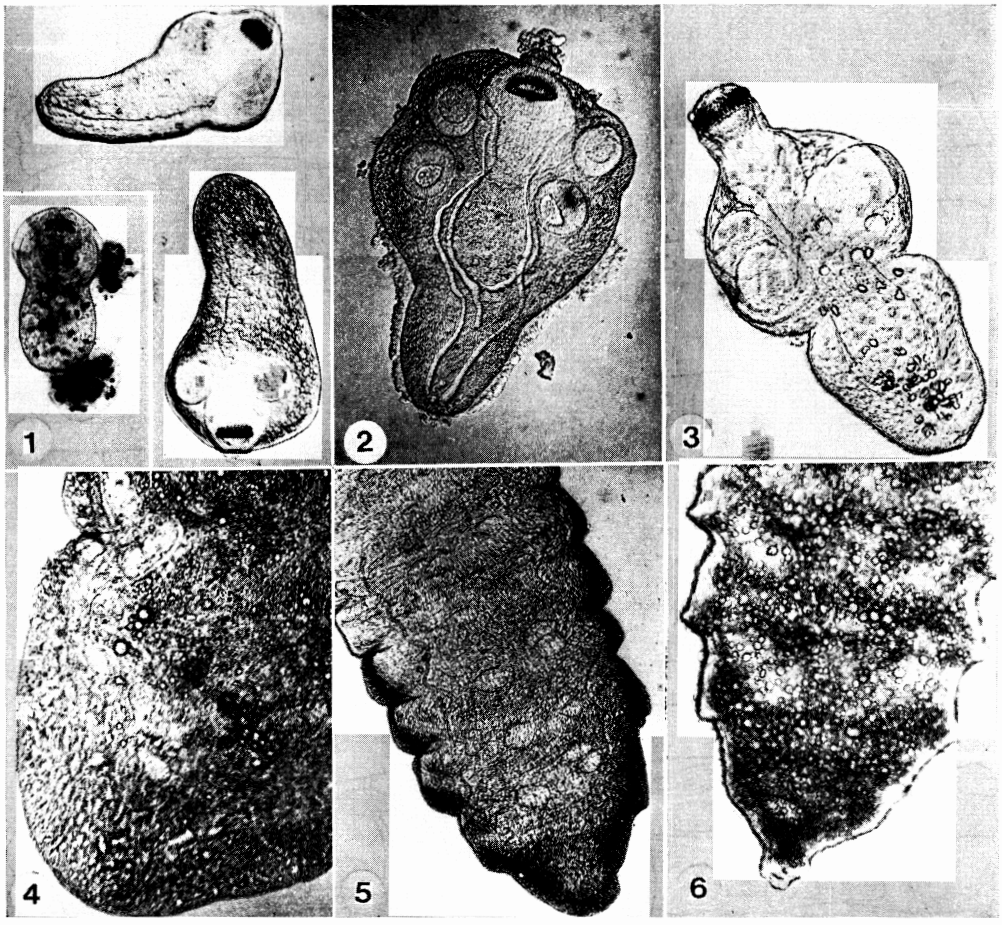

Explanation of Figures

Fig. 1 Two *in vitro* grown worms shown for comparison (in poor medium; 96 h post inoculation). Note the complete disappearance of corpuscles from one in the bottom while still few present in the worm on the top. Both worms were active when photographed (× 140). The inset figure is a trypsin excysted larva showing large numbes of corpuscles in the scolex, body and the reflected layer (× 140).

Fig. 2 An *in vitro* grown young adult (96 h old). Note the thick precipitate on the tegument and complete disappearance of corpuscles (× 220).

Eig. 3 An *in vitro* grown worm (in optimal medium; 96 h post inoculation). Note almost no reduction in number of corpuscles in the proglottidal part (× 220).

Fig. 4 A portion of the posterior third of a 9-day-old *in vitro* grown worm in the medium containing calf serum (Lot No. L 40239). Note the gradual disappearance of fat globules from the parenchyma and poor growth of the worm (× 140).

Fig. 5 Posterior third of an 11-day-old *in vitro* grown worm in the medium containing calf serum (Lot No. XVIII). Note the excretory canals and testes are clearly visible by the absence of parenchymal lipid globules. Precipitate on the tegument is almost absent (× 140).

Fig. 6 An *in vitro* grown 11-day-old worm; the medium contained horse serum (Lot No. Z 0239). Compare the concentration of lipids in the parenchyma with the Figs. 3 and 5 (× 140).

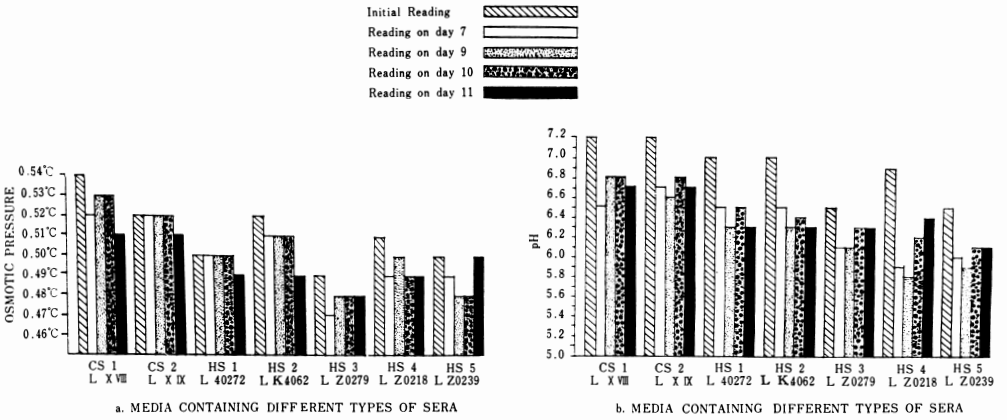

Fig. 7 Bar diagrams showing changes in osmotic pressure (a) and pH (b), respectively of different media during *in vitro* cultivation of *H. microstoma* from 4 day to 11 day (cf. Table 3).

sera could also be due to the presence of some cross reacting substances already formed in response to other previous parasitic or bacterial infections (Soulsby, 1962; Boyden, 1966). There is e.g. a possibility that grazing animals are more susceptible to acquire such substances than stall-fed ones. Tucker (1961; cited by Soulsby, 1962) has shown that a seasonal variation in the anti-R isoantibodies occurs in sheep on pasture but not in those animals which were housed. Soulsby (1962) also roported that functional antigens are shared by helminths at the oogenesis level. When sheep were immunized against *Cooperia* spp. and subsequently challenged with *Trichostrongylus* spp. the latter worms showed marked retardation in oogenesis which he believed to be due to cross reacting substances. From Table 3 it could be inferred that even among horse sera there appears to be a marked influence on oogenesis in experiments where the results regarding the two other criteria ("sperm in cirrus pouch" and "no of immature proglottids") were similar (compre results of HS-4 and HS-5).

In the preliminary experiments it has been observed that when worms were cultured from excysted stages, in some sera a thick precipitate is formed all over the tegument of the young adults (Fig. 2). In some other sera, however, the precipitate is much less (Fig. 3) or almost absent (Fig. 1). The precipitate is often pinkish or deep brown or brick red in colour. From Table 1, it will be further evident that such precipitate is formed during the early development phase (when 4-day-old young adults were cultured until day 11), that it either remains throughout the period of cultivation or gradually decreases with age, and that, except in some rare cases it does not altogether disappear (see Fig. 6). Damian (1964) and Capron *et al.* (1968), among others, believe that parasites in general would synthesise at their surface proteins which are antigenically similar to the host. They suggested that by doing so a parasite escapes recognition by the host. Smithes *et al.* (1969) similarly reported that hosts' glycoproteins are adsorbed to the surface of the parasites which thus mimic the host antigenically and avoid rejection. Of late, Bafus (1977) demonstrated that both *H. diminuta* and *H. microstoma* are capable of binding immunoglobulins of mouse to the tegument. As all the sera used in our experiments were pre-heated, the formation of precipitate on the tegument of *H. microstoma* might be the result of an interaction between the surface secretions by the parasite and substances present in the medium.

As previously supposed (Smyth, 1947; von Brand, 1952) lipids in cestodes are no

longer believed as absolutely an accumulation of waste products of metabolism. There appears to be a close relation between the development of *H. microstoma* and the lipid content of the media used. In fact, our *in vivo* studies also have indicated that all neutral fats are not waste products at least in this cestode (see Chowdhury and De Rycke, 1976 b).

In our prelminary experiments with different calf or horse sera, an interesting fact has been observed when worms were culturred from the excysted stages in a medium containing "bad serum". Normally the tapeworms in such a medium fail to grow, the corpuscles from these stages gradually disappear before the unstrobilated worms disintegrate in the medium showing abnormalities (compare inset in Fig. 1 with Figs. 1 and 2). This situation has been repeatedly noted irrespective of accumulation of precipitate on the tegument of the young adults. On the contrary, there is a rapid numerical insrease of corpuscles when the excysted stages were grown in a "good serum" (compare Fig. 2 with Fig. 3). This evidence supports our earlier postulation that worms utilise ingredients of corpuscles when they require these components (Chowdhury and De Rycke, 1974 b). The variability in the distribution of corpuscles of 11-day-old worms (cf. Table 4) is not only due to growth and development of the parasite but may also be related to the ion concentration and stimlatory effects of different sera. Stimulatory effects may also come from bile salts present in different sera (Haslewood, 1967; Chowdhury *et al.* 1974).

During the course of different experiments, it has been observed that *H. microstoma* is not only capable of withstanding an acid pH as low as 5.4 but also an osmotic pressure down to Δ=0.45 C. The worms under these circumstances continue to grow if the medium is containing a good serum. On the other hand, with a bad serum, worms fail to grow even at pH 7.2 (see also Smyth, 1974).

The effect of serum on growth, development and organogenesis of a metazoan parasite is definitely complex. As Shodell and Rubin (1970) remarked "serum is a complex mixture of lipids, hormones, enzymes and at least another sixty distinct proteins and peptides and cellular proliferation is a complex process, involving many different cellular activities". Clearly, concerted efforts are necessary to unveil the many problems.

Summary

H. microstoma has been cultured *in vitro* from 4 days to 11 days of age using Eagle's medium supplemented with lamb liver extract and one of seven different sera calf or horse. The experiments have shown that both growth and organogenesis are inhibited in the calf sera used. Although most of the horse sera supported growth, each serum stimulated development of worms to a varying degree. The worms grown in horse sera normally contain much neutral lipids while those grown in calf sera either contain very much less or none. The distribution of calcareous corpuscles in worms obtained from different culture appears to vary, which may be due to the ion concentration in the sera or their stimulatory effects.

It is felt that a suitable serum is necessary for optimal growth and organogenesis of the parasite *in vitro*. Besides poor neutral fat content, the inhibitory effects of certain sera possibly due to either lack of essential growth stimulating factors (discussed above) or presence of certain cross reacting substances most likely of parasitic and or microbial origin.

Acknowledgements

The senior author is grateful to Prof. Dr. G. Van Grembergen, Director, Laboratory of Zoophysiology, State University of Gent, for the space, facilities and encouragement to carry out this work. The research fellowship awarded by the Belgian Ministry of Education is also gratefully acknowledged.

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各種血清における培養 *Hymenolepis microstoma* の発育比較

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羊肝浸出液を加えた Eagle's medium に牛血清または馬血清を加えた計7種の培養液を用いた。それぞれ 5 ml ずつ廻転チューブにいれ，95% N_2+5% CO_2 で30秒間ガス処理し，約2～3時間 37.5 C に置いた後，予じめ Hank 液で4回洗滌した4日齢脱嚢虫体を投じ，さらに30秒間ガス処理して 37.5 C の孵卵器内の廻転ドラムに納めた．チューブ内のメジゥムは 7, 9, 10日で更新した．このようにして各培養基内での11日齢までの虫体の生長，器官の発達，外皮内沈澱物の形成，体肉内石灰小体の量的分布，中性脂質の蓄積などの状態を比較観察した．その結果，牛血清はいずれも虫体の生長と器官形成を妨害したが，馬血清間では虫体の器官の発達程度に差こそあれ促進作用を示した．牛血清中の虫体では中性脂質が極めて少ないか全く認められないが，馬血清ではいずれも多く認められた．虫体内石灰小体の分布は培養基間に差があり，血清中のイオン濃度または刺激作用のちがいによるものと思われるが，いずれにしても好適な血清が培養虫体の好適な生長と器官発達上必要なことが感じられる．血清の発育阻止作用としての虫体内中性脂肪含有量の過少は生長刺激要因の欠除または虫体と微生物的原因の両者またはいずれかによる交叉反応物質の存在によるものかと思われる．