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Further Observations on the *in Vitro* Development of *Hymenolepis microstoma* in Relation to the Lipid and Cholesterol Content of the Serum

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Introduction

From the gross observations and histochemical studies of *in vitro* grown *Hymenolepis microstoma*, from day 4 to day 11, it was observed that when these were grown in media containing different horse or calf sera, accumulation of fat globules in the parenchyma of young adults varied considerably. Later, it was realized that worms grown in media with calf serum normally contained less fatty acids than when grown in media prepared with horse serum (Chowdhury and De Rycke, 1979). These observations tempted to design simple experiments to study the growth and development of *H. microstoma* in the media containing calf and horse sera in relation to their total lipids and cholesterol contents.

Materials and Methods

The axenic cultivation technique used in the present study was as described by Evans (1970) and, Chowdhury and De Rycke (1979) with few modifications.

The basic medium consisted of main three parts: (a) Eagle's medium (= Basal Medium) (60%) purchased from Flow Laboratories; (b) liver extract (10%) prepared from the fresh lamb liver according to the method described by Sinha and Hopkins (1967); (c) serum (30%).

In this study two media have been prepared with two different specific sera: one of these being a horse serum (HS 5; HS 02; Lot No. Z0239) obtained from Wellcome Laboratories and the other was a calf serum (CS-1; Lot No. XVIII) received in bulk locally from the University of Louvain. It may be mentioned here that all the procedures adopted in the preparation of the media, changing of the media, distribution of the media in the roller tubes and the 'gas phase' were accomplished in the U.V. chamber in the sterile room. The Basal Medium used in the preparation of the two *final culture media* (FCM), were the same and, as usual in all our studies, had been sterilized by filtration after addition of glucose, $NaHCO_3$ and antibiotics. Stock serum and liver extract prepared in bulk were added to the basal medium prior to use for the preparation of FCM (Chowdhury and De Rycke, 1979). The FCM thus prepared was distributed in culture tubes and gassed with 5% CO_2 in 95% N_2 for

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Table 1 Concentration of total lipids and cholesterol in some of the sera used in the media for the *in vitro* cultivation of *H. microstoma*

Source	Code number of serum*	Total lipids in %	Cholesterol mg %
Obtained locally	CS-1† Lot No. XVIII	0.303	170
〃	CS-2 Lot No. XIX	0.489	160
Flow Laboratories	CS-3 Lot No. L40240	0.500	137
〃	CS-4 Lot No. L40304	0.800	155
〃	CS-5 Lot No. L40275	0.860	ND
〃	CS-6 Lot No. L40447	1.330	ND
〃	HS-1 Lot No. L40272	0.890	130
Wellcome Laboratories	HS-2 Lot No. K4062	1.144	110
〃	HS-3 Lot No. K4212	1.199	85
〃	HS-4 Lot No. K4308	1.090	95
〃	HS-5 Lot No. K2911	1.200	ND
〃	HS-6 Lot No. K4068	1.240	ND
〃	HS-7 Lot No. K5428	1.203	100
〃	HS-8 Lot No. Z0279 (HS-02, No. 5)	0.680	ND
〃	HS-9† Lot No. Z0239 (HS-02, No. 5)	0.750	90
〃	HS-10 Lot No. Z0218 (HS-02, No. 5)	0.810	ND

* CS=calf serum HS=horse serum
ND=estimation not done
† Sera used in the present experiments

1 minute. The experiments were repeated three times and the initial pH, which was always 6.9 and osmotic pressure (Δ=0.54 C) were recorded after gassing.

The 4-day-old young adults of *H. microstoma* were cultured in the above media until these became 11-day-old. The culture medium was changed on days 7, 9 and 10. In these series of experiments half of the worms grown in the medium containing horse serum were transferred to the other medium containing calf serum on day 7 and, the half of the worms grown in the medium with calf serum were transferred to the medium prepared with horse serum on day 9 (Tables 2, 3). The 'transfer' of worms, eventually, was determined from several preliminary experiments. Before transferring into the different media, the worms were washed with Hanks' solution (prepared also in bulk) at least 4 times.

For the evaluation of results obtained from cultivation experiments in each of the media, 25 largest worms from three periments (from a total of 45 wor cultured per medium) were used (Tab 2, 3). Of the selected 25 worms, t length measurements were taken from t 20 worms (Table 2). The distribution neutral lipids and phospholipids we studied histochemically using Sudan Bla B, Sudan IV and, copper phthalocyani methods for staining. For details of hist chemical methods employed for studies calcareous corpuscles and lipids, reader referred to our previous papers (Chowdhu and De Rycke, 1974a; 1976).

To understand better the growth an organogenesis in worms in relation to th lipids and cholesterol content of the sera, total of 10 and 15 samples of different sera (including the two specific sera used in the present experiment) were respectively analyzed (Table 1). The cholesterol content was estimated spectrophotometrically, while the total lipids extracted with methanol was determined gravimetrically.

Results

The comparative growth and development of *H. microstoma*: (a) from 4 to 11 days of age in the media containing specific horse serum and calf serum, and (b) by replacing worms from horse-serum medium to calf-serum medium and from calf-

Table 2 Effects of media with two different sera on the growth and organogenesis of 11-day-old *Hymenolepis microstoma*, grown *in vitro* from 4-day-old young adults (cf. Table 1; Plate I, Figs. a-d)

(Summary of three experiments)

Serial number of worms checked	Code number of serum used in the medium and duration of culture											
	HS-5; Lot No. Z0239 (day 4-11)			CS-1; Lot No. XVIII (day 4-11)			HS-5; Lot No. Z0239 (day 4-7) CS-1; Lot No. XVIII (day 7-11)			CS-1; Lot No. XVIII (day 4-9) HS-5; Lot No. Z0239 (day 9-11)		
	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
1	72	30	149	—	—	—	36	18	56	—	—	30
2	56	28	92	—	—	—	—	—	73	—	—	43
3	34	21	85	—	—	—	—	—	84	—	—	39
4	48	11	105	—	—	—	—	—	83	7	4	36
5	27	27	49	—	—	—	32	6	84	—	—	17
6	100	27	153	—	—	—	15	2	91	—	—	47
7	72	13	93	—	—	—	8	4	63	—	—	54
8	113	23	141	—	—	—	—	—	71	—	—	37
9	80	25	112	—	—	—	16	10	77	—	—	—
10	52	32	70	—	—	—	20	12	98	—	—	—
Mean	65.4	23.7	104.9	—	—	—	12.7	5.2	78.0	0.7	0.4	30.0
Average length(n=20) in mm (±SE)	33.90±1.714			8.7±0.490			12.80±0.780			10.50±0.340		

* CP=Cirrus Pouch
LS=Lateral Sacs
IT=Proglottids with immature testes

serum medium to horse-serum medium, are summarized in the Table 2. From the same Table and Fig. b in the Plate I, it could be inferred that when worms were transferred on day 7 from the medium containing the horse serum to the one with the calf serum, both the general growth and development of gonads were severely affected. By transferring the worms grown in the calf-serum medium to the horse-serum medium on day 9, marked improvement is noted in all respects (Table 2, Fig. d in Plate 1). The relative growth and development in all of *in vitro* grown worms can also be compared with the *in vivo* grown worms of the same age (cf. Plate I).

There appears to be a parallelism in the distribution of calcareous corpuscles in these worms and their growth and development in the media (compare results of Table 3 with Table 2).

The distinct histological (also histochemical) difference in the concentration of neutral lipids in the worms grown in the media containing specific horse or calf serum has been depicted in the Figs. 1 and 3. When worms from the horse-serum medium were transferred to the calf-serum medium on day 7, there was a marked reduction in fats in the worms (Fig. 2). Similarly, by transferring the worms from the calf-serum medium to the horse-serum medium on day 9, neutral lipids begin to appear in the parenchyma of the worms

Table 3 Effects of media with two different sera on the quantitative distribution of calcareous corpuscles* of 11-day-old *H. microstoma*, grown *in vitro* from 4-day-old young adults (cf. Table 2)

Serial number of proglottids†	Code number of serum			
	HS-5 (day 4-11)	CS-1 (day 4-11)	HS-5 (day 4-7) CS-1 (day 7-11)	CS-1 (day 4-9) HS-5 (day 9-11)
1	48.1	1.8	33.7	5.1
2	69.8	3.4	33.8	7.0
3	65.5	1.7	32.5	9.2
4	78·2	2.5	36.7	10.3
5	78.1	2.7	39.5	11.0
6	75.8	3.4	36.7	11.4
7	81.2	2.7	45.7	10.0
8	72.2	3.2	40.4	12.4
9	79.4	3.4	45.5	12.0
10	83.0	5.1	45.0	10.1
Totals	731.3	29.9	389.5	98.5

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

† The second proglottid, next to the end-proglottid, represents the first proglottid. Each figure is the average of determinations on 7 different worms.

on the following day (Fig. 4) and on day 11 accumulation of lipids (Fig. 5) become parallel to 7 or 8-day-old *in vivo* grown worms. All cultured worms contained phospholipids in calcareous corpuscles as in *in vivo* grown worms. As expected, in the distribution of phospholipids in the parenchyma and reproductive organs, when comparing similar stages in *in vivo* development (Cowdhury and De Rycke, 1976), no difference can be observed.

In these experiments, the maximum change in pH was observed on day 7 (4-11 days) of culture which were respectively 5.9 (horse-serum medium) and 6.1 (calf-serum medium). And, after changing the worms, the maximum fall was noticed on day 10 which were respectively 6 in horse-serum medium (9-11 days) and 6.2 in calf-serum medium (7-11 days). This indicating better metabolism of worms in the medium containing horse serum. Apparently no difference could be observed in the osmotic pressure of different media.

Discussion

From the results presented in the Table 1, it could be inferred that there is a marked difference in the concentrations of total lipids and cholesterol in different sera. Many of the horse sera analyzed appear to contain a higher percentage of total lipids than the calf sera while the concentration of cholesterol is much higher in the calf sera than in the horse sera.

The significance of neutral lipids and cholesterol in the *in vitro* development of *H. microstoma* is rather difficult to explain at the moment, since lipid and cholesterol metabolism in cestodes is poorly understood (discussed in detail by von Brand, 1973). In our *in vivo* studies with this cestode (Chowdhury and De Rycke, 1976), we have,

however, indicated that some amounts of lipids would be required for the maturation of gonads and transformation of the fertilized ova to the oncosphere. From the Table 1, it is now known that the horse serum- HS 5 (HS 02; Lot No. Z0239) used in the present experiment in the preparation of the horse-serum medium contains almost double the concentration of total lipids as compared to the calf-serum medium containing the calf serum- CS I (Lot No. XVIII). With regard to the cholesterol, there is nearly a reverse situation in these two specific sera—the calf serum containing much higher concentration of it.

Serum is a complex substance containing various "growth stimulating factors" beside lipids and is essential for the axenic culture medium component, which has been explained earlier by us (Chowdhury and De Rycke, 1979). That these factors either individually or collectively could have influenced the development of the cestode can not be ruled out. It is, however, obvious from the text that we have made the two specific culture media apparently constant by preparing different ingredients in bulk excepting the sera. The results of the repeated experiments were always consistent. Further, the specific sera were also used in other occasions by us and other workers (see Khan and De Rycke, 1975) in the laboratory and the results of these experiments corroborate the present findings. Hence, it could be presumed that lipids and cholesterol content of these sera might have played some role in the organogenesis of the tapeworm. The present experiments have given a clear indication to that direction. Certainly, further experiments are necessary to confirm these promise.

The factor(s) which may influence the variability in the quantitative distribution of calcareous corpuscles in worms could be: (a) concentration of important ions in the medium, (b) stimulatory effects of the medium due to the presence of bile salts in the serum (Haslewood, 1967; Chowdhury *et al.*, 1974).

It is tempting to point out here that Shield (1969) has ably demonstrated cholinesterase in the corpuscles of *Dipylidium caninum* which possibly did not receive their attention. In cestodes (*H. diminuta*) its presence in the embryo has been suggested to be linked with embryonic muscle formation (Rybicka, 1967). While in trematodes, Edwards *et al.* (1971), Bueding and Bennett (1972) and Nizami *et al.* (1977) maintained that this enzyme is involved either in "biochemical adaptation", immune response or in neurotransmission. Since "corpuscles" is a complex and fundamental system, localization of cholinesterase in numerous concretions in *H. microstoma* (Chowdhury and De Rycke, 1974b) and possibly in many other cestodes, has not received due attention. At this stage we possibly can not discount altogether if this specific enzyme has got any significance in cestode's fat metabolism and/or translocation.

Summary

Hymenolepis microstoma has been cultured *in vitro* from 4 days to 11 days of age using Eagle's medium supplemented with lamb liver extract and a serum—horse or calf. The experiments have shown that worms grow better in the medium containing horse serum ("good medium") rich in lipids and poor in cholesterol than in the medium prepared with calf serum ("bad medium") with low lipid and high cholesterol content. When the worms are transferred from "good medium" to "bad medium", organogenesis in them is severely affected but growth and development improves when young cestodes are removed from the culture medium with the calf serum to one with horse serum. These situations are always found consistent with the accumulation of parenchymal lipids in

worms. These results together with analysis of various sera used in different experiments have apparently indicated that growth and development of young adults are related to the concentration of lipids and cholesterol in the sera used. However, the role of other factor(s) in the growth and organogenesis of worms in the complex media can not be ruled out, which has been discussed.

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Hymenolepis microstoma の *in vitro* 発育に関するその後の研究：血清中の脂質およびコレステロール含量の与える影響

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4～8 日齢の *H. microstoma* を小羊肝抽出液と馬又は牛の血清とを添加したイーグル培養液内で培養した．その結果，低脂質・高コレステロールの牛血清（不良培養液）に比較して高脂質・低コレステロールの馬血清（良好培養液）において虫体の発育が優れていた．良好培養液から不良培養液へと虫体を移すと器官発生は強い障害を受けた．しかし幼条虫を牛血清含有培養液から馬血清含有培養液に移すと成長，発育は改善された．このような現象は虫体実質内での脂質の蓄積とよく一致していた．これらの成績と種々のロットの血清の分析結果とを併せ考えると，幼若成虫の成長・発育は用いた血清中の脂質・コレステロール含量に関連していることが示唆された．しかし条虫の発育や器官形成に及ぼす他の因子の存在も否定できない点についても考察を加えた．

Explanation of Figures

Plate I (Figs. a–d)

Illustrations of organogenesis in 11-day-old *Hymenolepis microstoma,* grown *in vitro* from 4–11 days (cf. Table II). The figure on the right side is from an 11-day-old *in vivo* grown worm for comparison (all figures are drawn approximately from the same region of the worms).

Fig. a Proglottids of a worm grown *in vitro* (day 4–11) in a medium containing horse serum No. HS-5 (Lot No. Z0239)

Fig. b Proglottids of a worm grown *in vitro* (day 4–11) first cultured in a medium containing horse serum No. HS-5 (Lot No. Z0239) and then (day 7–11) in a medium containing calf serum No. CS-1 (Lot No. XVIII)

Fig. c Proglottids of worm grown *in vitro* (day 4–11) in a medium containing calf serum No. CS-1 (Lot No. XVIII)

Fig. d Proglottids of a worm grown *in vitro* (day 4–11) first grown (day 4–9) in a medium containing calf serum No. CS-1 (Lot No. XVIII) and then (day 9–11) in a medium containing horse serum No. HS-5 (Lot No. Z0239)

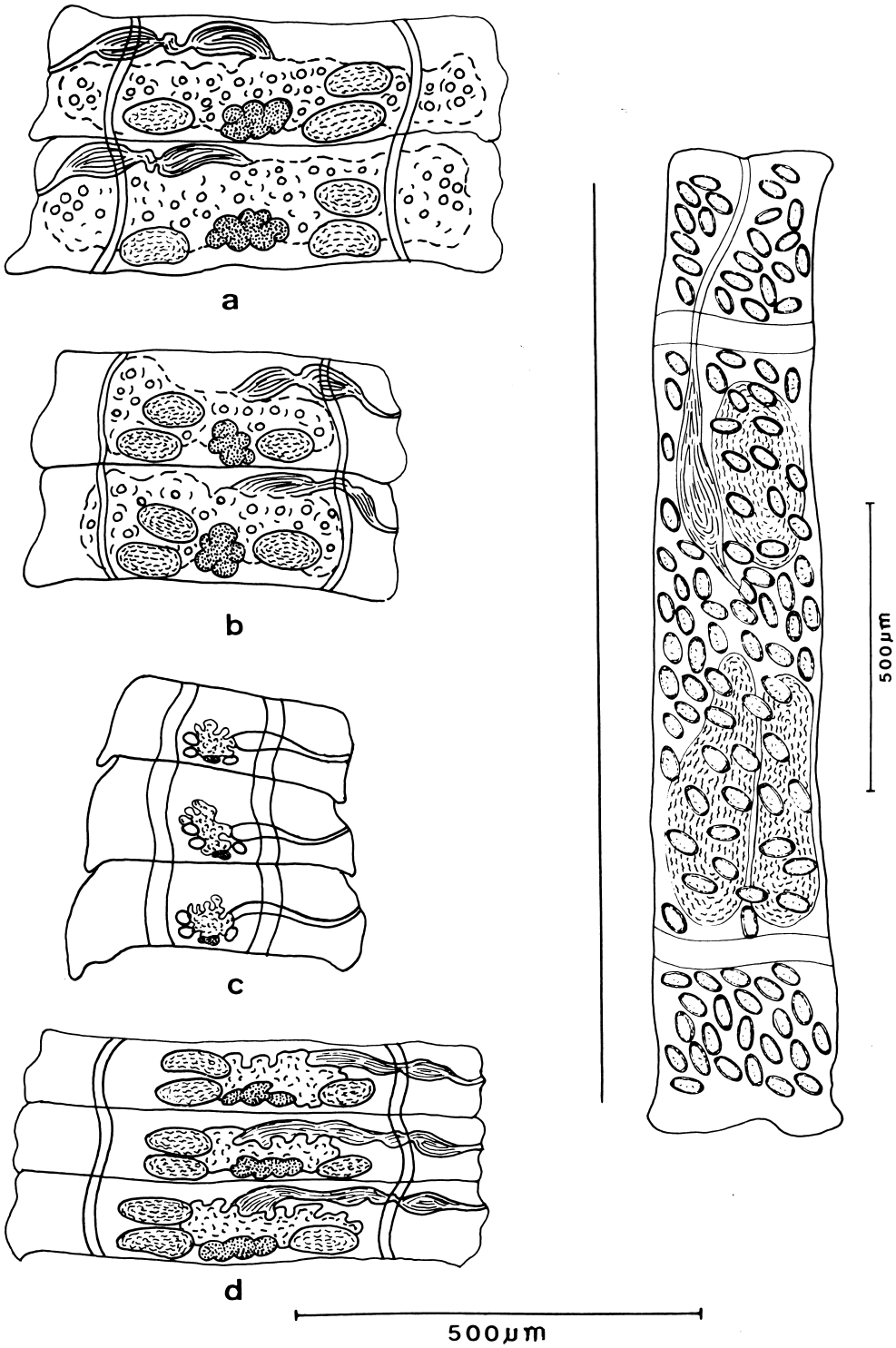
Fig. 1 Posterior third of an 11-day-old *in vitro* grown *Hymenolepis microstoma*; the medium contained horse serum No. HS-5 (Lot No. Z0239). Note the accumulation of fats in the parenchyma. ×140

Fig. 2 An *in vitro* grown 11-day-old worm (posterior third); first cultured (day 4–7) in a medium containing horse serum No. HS-5 (Lot No. Z0239) and then (day 7–11) in a medium containing calf serum No. CS-1 (Lot No. XVIII). Note the fertilized ova in the lateral sacs (LS), calcareous corpuscles (CC) and excretory canals (EC) are clearly discernible in the region. Compare this with the Fig. 1. ×140

Fig. 3 Posterior third of an 11-day-old *in vitro* grown *H. microstoma*; the medium contained calf serum No. CS-1 (Lot No. XVIII). Note the parenchyma devoid of fat globuels. Also observe testes (T), corpuscles (CC) and excretory canals (EC) in the parenchyma. Compare this Fig. with Figs. 1 and 2. ×140

Fig. 4 10-day-old worm (posterior third); first cultured (day 4–9) in a meduim containing calf serum No. CS-1 (Lot No. XVIII) and then (day 9–11) in a medium containing horse serum No. HS-5 (Lot No. Z0239). Note the begining of accumulation of lipids in the parenchyma. Excretory canals and corpuscles are still visible. ×140

Fig. 5 Posterior third of an 11-day-old worm as cultured above. Note the lipid globules again masking all structures in the parenchyma as in Fig. 1. ×140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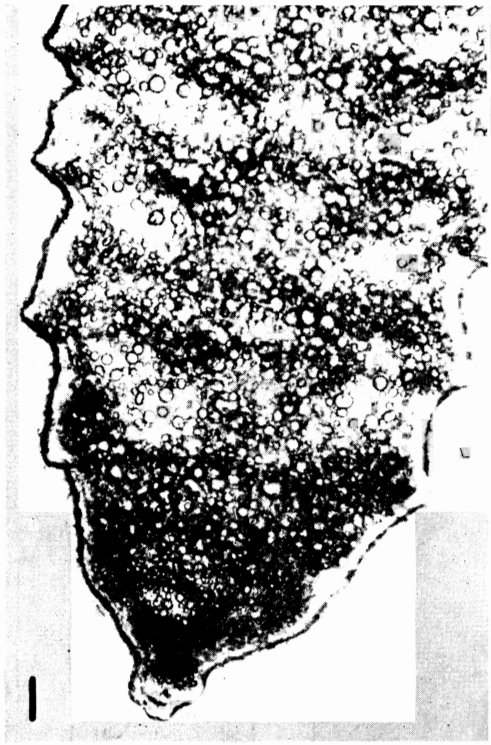

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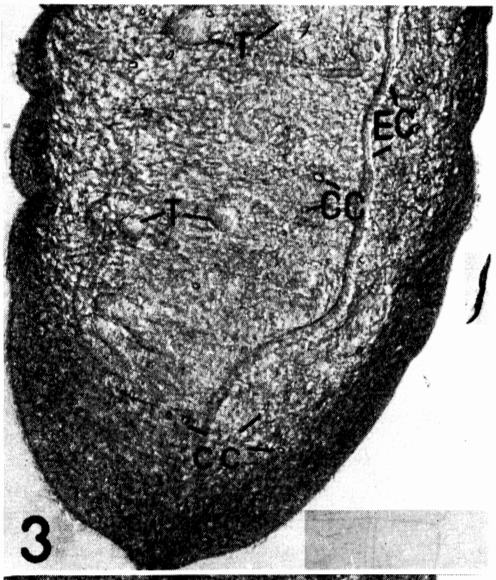

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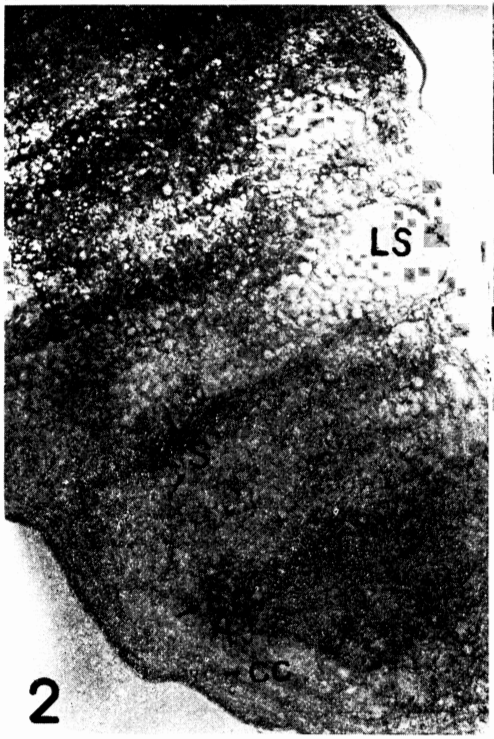

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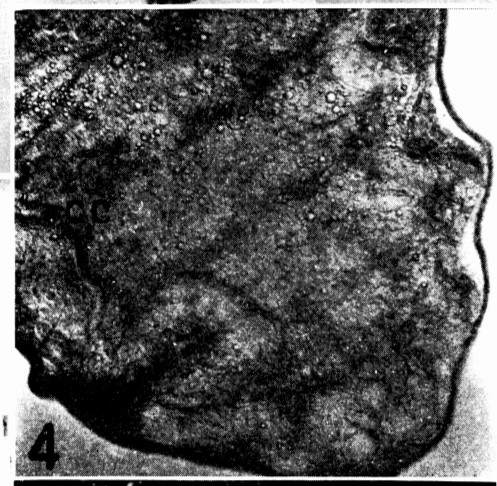

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