STUDIES ON TAPEWORM PHYSIOLOGY

III. ASEPTIC CULTIVATION OF LARVAL DIPHYLLOBOTHRIIDAE IN VITRO

By J. D. SMYTH, Department of Zoology, Trinity College, Dublin, Eire

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(With Plate 8 and Three Text-figures)

INTRODUCTION

The difficulty of keeping cestodes alive outside the host body for periods long enough for metabolic determinations to be carried out has been mainly responsible for the absence of detailed knowledge of the physiology of these helminths. The main problem has been a technical one, and centres on the fact that aseptic conditions are essential for successful cultivation. The starting-point in culture attempts must therefore be directed primarily towards the attainment of asepsis and parasitologists 'will have to adopt a technique more akin to that of the bacteriologist than that of the physiologist' (Wardle, 1937).

Following this line of approach, it has recently been shown (Smyth, 1946, 1947a) that by commencing with the larval rather than the adult cestode phase, the pseudophyllidean cestodes Schistocephalus solidus and Ligula intestinalis can be cultured to sexual maturity in vitro. Since the larval stages (plerocercoids) of the above-named species occur in the coelomic cavity of fish (i.e. a natural aseptic environment)—the problem of initial microfloral contamination does not arise. At room temperatures, plerocercoids of Schistocephalus have been kept alive in peptone-broth for periods up to 300 days. Adult worms of both Ligula and Schistocephalus were obtained from the plerocercoids in vitro by raising the temperature of cultivation to 40°C. (i.e. the body temperature of birds which form the definitive hosts for these species of cestodes). The adults so produced were apparently normal histologically, but the eggs produced in vitro were found to be infertile. It is evident that a similar technique can be applied to the plerocercoid larvae of any cestode found in the coelomic cavity of fish—and there are a great many of such forms known.

In the case of Ligula and Schistocephalus, the plerocercoids are already of considerable size when removed from the fish and the rudiments of the genitalia are present and well defined; the problem of inducing larvae to become sexually mature in vitro is therefore comparatively simple. The plerocercoids of many other cestodes, however, are very minute and never develop the anlagen of the genitalia while in the cold-blooded host. In such forms, considerable growth must take place before the genitalia develop and the sexually mature worms are formed. The present experiments deal with a minute larva of this type, and their objective has
been to determine whether: (a) the technique used for obtaining aseptic cultures of large forms can be successfully applied to minute forms; (b) the media in which *Ligula* and *Schistocephalus* become sexually mature are suitable for growth and development of such minute larvae.

The previous literature on culture attempts *in vitro* has recently been reviewed in detail elsewhere (Smyth, 1947b) and will not be given here.

**MATERIAL**

The larvae used were those of an unidentified pseudophyllidean cestode from the body cavity of *Gasterosteus aculeatus*. The fish were obtained from Thryberg Reservoir, near Rotherham, Yorkshire. Specimens were collected by means of traps, rod and line, and nets. About 90% of the fish examined were infected. Plerocercoids occurred both free and encysted in many sites in the body cavity of the fish—the liver being the most frequently and the most heavily infected.

It was not possible to identify the species with any degree of certainty. The size range was 1–10 mm. long and 0.5–2.0 mm. wide. The scolex was invaginated in larvae freshly removed from the fish (Pl. 8, fig. 2), but became evaginated when the organisms were relaxed in tap water at 40 °C. for a few minutes (Pl. 8, fig. 3). The relaxed and evaginated scolex (Pl. 8, fig. 1) was of the typical pseudophyllidean type with very deep lateral bothria. The outline of the scolex was triangular or oval but showed great variation depending on the degree of relaxation. Externally, the surface was rugose in appearance, but this condition was probably only superficial and it is doubtful whether any true segmentation was present.

The presence of lateral bothria places the cestode in the family Diphyllobothriidae. The above description agrees closely with that of the plerocercoids of *Diphyllobothrium ditremum* and *D. dendriticum* which have been reported from freshwater fish in Eire (Harris & Hickey, 1945); the adults of these species occur in gulls. The reservoir at Thryberg is frequented by numerous gulls and other fish-eating birds, and it is possible that the larvae used in the present experiments may be those of one or both of the above-named species. Satisfactory identification, however, can only be based on detailed examination of sexually mature proglottids, and since these were not available for examination, it would be unwise to speculate further on possible identification without additional evidence.

**TECHNIQUE FOR ASEPTIC CULTIVATION**

The technique used for aseptically removing the larvae from infected fish was essentially similar to that described for *Schistocephalus* and *Ligula* (Smyth, 1946; 1947a) with further modifications and improvements. The specimens of fish were considerably smaller (3–4 cm. long) than those used for the work on *Schistocephalus*, and some difficulty was encountered in devising a satisfactory method for holding the fish securely during dissection. The procedure can be conveniently described in the form of instructions, which are given below:

1. Holding the fish lightly between the fingers and taking due care not to rupture the body wall, kill it by pithing with a fine needle.

2. Dry the fish with a soft cloth, and cut away the paired fins.
376 J. D. Smyth

(3) Grip the skull firmly, but carefully, with a pair of large-toothed forceps (Text-fig. 1, FL) and clamp these forceps in a retort clamp. Grip the tail with a second (smaller) pair of forceps (Text-fig. 1, FS) and clamp these forceps in another clamp in an adjacent retort stand. The fish should now be firmly held in a horizontal position with the ventral surface facing the operator.

(4) Place a strong light slightly to the left of the operator and arrange it to shine on to the surface of the fish. (It is difficult to locate minute larvae deep within the body cavity unless good illumination is used.)

(5) Sterilize the surface of the skin by painting with a coat (or two if necessary) of iodine (saturated solution in absolute alcohol) and allow to dry.

(6) While the iodine is drying, remove the dissecting instruments from the sterilizer and place in a convenient position near the right hand.

(7) Make a median anterior incision in the body cavity with a fine cornea knife, and continue the cut backwards to the pelvic girdle. Cut through the pelvic girdle with fine scissors (it is almost impossible to do this with the cornea knife alone, as the strain produced is sufficient to tear apart the body from the skull). Continue the median incision with the knife to within a few millimetres of the anus. Grip the upper skin flap with a fine forceps and cut it away with the scissors.

(8) Using a flamed platinum loop in the right hand, pick up the minute larvae lying free in the body cavity and transfer to the culture tube held in the left hand. The cotton-wool plug can be conveniently removed with the little finger of the right hand, following the usual bacteriological procedure.

(9) For the removal of encysted larvae it is convenient to have the culture-tube clamped as shown in Text-fig. 1; both hands are thus left free. The larvae are freed by teasing away the host capsule with the cornea knife (left hand) while pressing lightly on it with the loop (right hand).

The above procedure requires some practice but is easily mastered. It is advisable to carry out the actual dissection in a room which is as free from draughts as possible, in order to reduce the possibility of contamination from air-borne spores of micro-organisms. Ideally, a sterile room is indicated. Apart from the maintenance of stringent bacteriological procedure, the most important controlling factors in the attainment of successful aseptic cultures are rapidity of dissection and manipulation.

Larvae were cultured in plugged rimless bacteriological culture tubes containing 10 c.c. of medium. The behaviour of the larvae was examined periodically by means of the reflecting microscope arrangement described previously for Schistocephalus. The present experiments were essentially directed towards the establishment of a satisfactory aseptic technique and only a small range of media was used. The basis of the nutrient media used was the commercial meat extract known as ‘Lab-Lemco’ (prepared for bacteriological purposes by Messrs Oxo Ltd., London) but other nutritional substances—glucose, yeast, serum—were used in addition. A 1% solution of Lab-Lemco in 0.5% sodium chloride formed the basic ‘broth’. The saline media employed was ⅓ Locke’s saline (referred to as ⅓ Locke). Previous work (Smyth, 1946) has shown that this dilution is approximately isotonic with Schisto-
Studies on tapeworm physiology

cephalus which is also parasitic in the coelomic cavity of Gasterosteus; and it seemed reasonable to conclude that a similar concentration would also be isotonic with larval Diphyllobothriidae from the same host.

Culture tubes containing media were incubated at 40°C for 48 hr. before use in order to eliminate any accidentally infected. The tubes were removed from the incubator just prior to the dissection of the fish, so that larvae commenced their period of cultivation in warm media; when the cultures were prepared they were returned to the incubator immediately.

![Text-fig. 1. Arrangement of apparatus used during aseptic dissection of fish. Both retort stands are clamped securely to the bench. C, clamps; FL, large-toothed forceps; FS, small forceps; G, G-clamp; K, cornea knife; R1, R2, retort stands; S, fish with paired fins removed; T, culture tube.]

The viability of the larvae at room temperatures was not determined in detail. Of eight larvae kept in broth + 1% peptone + 0.5% glucose at room temperatures, seven still showed active undulation on exposure to light after 80 days' cultivation.

EXPERIMENTAL RESULTS

Results of cultivation experiments are given in Table 1. Duration of normal behaviour was taken as an approximate criterion for the suitability of the culture media. 'Normal behaviour' may be defined as that period during which the larvae exhibit active movement of the scolex and strobila. The end of the period of normal behaviour was characterized, in the majority of larvae, by the loss of activity and subsequent degeneration of the scolex; in larvae dying without degeneration (most
saline media) the appearance of abnormal swelling, twisting or tetanus of the organisms was taken as indicating the end of the normal behaviour period.

In nutrient media, most larvae remained active for 2-7 days after degeneration had commenced; during this post-degeneration period, the non-degenerating region continued to undulate in a very normal manner. Larvae usually died when about half the strobila had undergone degeneration. On the average, large larvae had a longer post-degeneration period than small ones, whereas the duration of normal behaviour (as far as could be judged purely by observation) was apparently independent of the size. For this reason the duration of normal behaviour can be considered to be a more reliable (but still very approximate) criterion than the total viability.

<table>
<thead>
<tr>
<th>Ref. no. of exp.</th>
<th>No. of larvae used</th>
<th>No. of sterile cultures</th>
<th>% asepsis</th>
<th>Medium</th>
<th>Viability in days</th>
<th>Period of normal behaviour in days</th>
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<tr>
<td>38</td>
<td>8</td>
<td>5</td>
<td>62%</td>
<td>Locke</td>
<td>2</td>
<td>4</td>
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<tr>
<td>62A</td>
<td>5</td>
<td>5</td>
<td>62%</td>
<td>Locke</td>
<td>4</td>
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<tr>
<td>64</td>
<td>10</td>
<td>10</td>
<td>66%</td>
<td>Locke+0.25% glucose</td>
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<td>2</td>
</tr>
<tr>
<td>68</td>
<td>4</td>
<td>5</td>
<td>55%</td>
<td>Locke+0.5% glucose</td>
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<td>2</td>
</tr>
<tr>
<td>69</td>
<td>5</td>
<td>5</td>
<td>83%</td>
<td>Locke+1% glucose</td>
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<td>0</td>
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<tr>
<td>67</td>
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<td>10</td>
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<td>2</td>
</tr>
<tr>
<td>24B</td>
<td>5</td>
<td>5</td>
<td>75%</td>
<td>Broth + 1% peptone</td>
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<td>53</td>
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<td>4</td>
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<td>6</td>
</tr>
<tr>
<td>71</td>
<td>6</td>
<td>6</td>
<td>100%</td>
<td>Broth + 1% peptone +10% glucose</td>
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<td>4</td>
</tr>
<tr>
<td>59</td>
<td>10</td>
<td>10</td>
<td>100%</td>
<td>Broth + 1% peptone +0.5% glucose +0.5% yeast</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>62B</td>
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<td>broth + 0.75% peptone</td>
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<tr>
<td>40</td>
<td>10</td>
<td>9</td>
<td>90%</td>
<td>Broth + 0.75% glucose</td>
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<td>2</td>
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<tr>
<td>30</td>
<td>6</td>
<td>4</td>
<td>66%</td>
<td>Broth + 10% horse serum</td>
<td>9</td>
<td>12</td>
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</table>

The duration of normal behaviour showed some variation for individual larvae even in the same media, but—excepting Exp. 69 (1/2 Locke + 1% glucose)—the variation was small enough to allow statistical methods to be applied. Differences in the duration of normal behaviour periods in different media were considered significant if \( \frac{M_1 - M_2}{\sqrt{[\sigma_{M_1}^2 + \sigma_{M_2}^2]}} \) was greater than 2.

A detailed account of the behaviour in the various media is given below.

**Nutrient media.** Activity in pure broth was markedly sluggish, the maximum normal behaviour period being 2 days, and the maximum viability 3 days. The addition of peptone greatly increased the activity, and the behaviour figures in
Studies on tapeworm physiology

Peptone-broth and three-quarter strength peptone-broth (max. = 7 days) are significantly higher than those of pure broth. The addition of yeast or glucose to peptone-broth did not appreciably affect the observable activity or significantly influence the normal behaviour times. Activity and general behaviour was most marked in serum-broth, and larvae in this media showed periods of normal behaviour (9–10 days) very significantly higher than those of any other nutrient media.

The behaviour of the larvae prior to the onset of degeneration was essentially the same in all nutrient media apart from the differences in the degree of activity as noted above. Immediately on immersion in the warm media, larvae became extremely active and underwent rapid expansion and contraction. Within an hour the vesicular membrane along the entire length became wrinkled in appearance and was gradually shed off within the next 12 hr. The membrane was cast off first from the scolex end, and the freeing of the membrane from this end was accompanied by evagination of the invaginated scolex. The evaginated scolex showed very marked undulant movement and the movements of the bothrial lips were plainly observed. After some hours larvae became attached to the walls of the tube by their bothria—one bothrium only effecting attachment. Some larvae even became attached to strobila of other larvae in the same tube, or to their own strobila by folding backwards. When attached the lips of each bothrium became greatly expanded in a remarkable manner so that when viewed through the tube walls with the reflecting microscope, the bothrium gave the appearance of a leech-like sucker almost circular.

Text-figs. 2, 3. Scolex of larva, during cultivation in vitro, showing bothrial lips drawn out to form a large sucker firmly attached to walls of culture tube. Text-fig. 2, as seen from below, through wall of culture tube. Text-fig. 3, as seen from the side. L, bothrial lips; S, strobila; T, wall of culture tube.
in outline (Text-figs. 2, 3). The bothria obtained a very firm hold on the tube walls and considerable shaking was necessary to free attached larvae.

As cultivation continued the strobila became considerably lengthened and so thin that the excretory canals and even the minute calcareous corpuscles were easily discernible by reflected light. As the larvae lengthened faint cross-striations became visible; these later became more distinct, and larvae surviving for 5 days or more showed definite segmentation.

The approach of degeneration was signalled by the slowing down of undulant movement of the scolex. Within a few hours, this movement ceased completely and the scolex became opaque and limp. The degeneration then progressed from the scolex backwards until the death of the larva.

**Saline media.** In pure $\frac{1}{3}$ Locke, behaviour and activity for the first 1–3 days was not significantly different from that in peptone-broth, most larvae becoming attached to the walls of the tubes in the manner described above. Activity soon fell off and larvae became contracted and died rapidly—the majority of larvae showing no typical degeneration of the scolex, although posterior degeneration sometimes occurred. The addition of glucose in concentrations of 1 or $0.5\%$ had a highly significant effect on the behaviour and viability. Activity in $\frac{1}{3}$ Locke+$0.5\%$ glucose was considerably less than in pure $\frac{1}{3}$ Locke, few larvae became evaginated and no bothrial attachment occurred; all larvae were contracted and abnormal after 2 days' cultivation and died within 3 days. $\frac{1}{3}$ Locke+$1\%$ glucose was toxic to the larvae and behaviour in this medium was very abnormal. All larvae showed abnormal behaviour within 24 hr.; four of the five larvae died within 48 hr. but one larva survived for 4 days. In $\frac{1}{3}$ Locke+$0.25\%$ glucose, the activity was comparable to that in pure $\frac{1}{3}$ Locke but the behaviour figures were significantly lower and identical with those for $\frac{1}{3}$ Locke+$0.5\%$ glucose; attachment to the tube walls never occurred although the scolex evaginated normally.

In both saline and nutrient media, no significant pH change was recorded after cultivation.

**MICROSCOPICAL OBSERVATIONS**

(1) **General histology**

Pieces of non-degenerating regions of the larvae were fixed after cultivation for varying times in the following media: pure broth, peptone-broth, glucose-broth, $\frac{1}{3}$ Locke, $\frac{1}{3}$ Locke+$0.5\%$ glucose.

Histological examination revealed that no somatic growth had occurred during cultivation; no evidence of mitosis was observed in any of the nuclei in any part of the strobila. Although some larvae showed small accumulations of nuclei in the central region of the parenchyma it is questionable whether these can be taken to represent the developing anlagen of the genitalia. The area of transverse section of cultured larvae was considerably smaller than those of fresh uncultured forms—an effect due to the marked elongation during cultivation. The excretory system showed marked changes. In fresh larvae, the lumen of the lateral excretory canals—which lie for the most part around the periphery of the section—are small (Pl. 8,
Studies on tapeworm physiology

fig. 4). In cultured larvae, the excretory canals are clearly visible as large oval or circular spaces (Pl. 8, fig. 5, E) which fill about one-quarter the area of the section. The two central excretory canals (Pl. 8, fig. 7, EC) undergo no marked change after cultivation.

(2) Distribution of fat

Material was fixed in formol-saline, imbedded in gelatine. Frozen sections were prepared and stained with Sudan Black following the usual technique. The handling and staining of frozen sections of such minute forms—even with the aid of a dissecting microscope—is one of very considerable difficulty, and sections were invariably wrinkled or slightly damaged, although the quantity of the fat present was adequately demonstrated (Pl. 8, fig. 6).

(a) Fresh uncultured larvae. Sections were almost devoid of fat. Minute droplets were occasionally seen under oil immersion. The vesicular membrane enclosing the larva stained a faint blue-black. Owing to the very minute quantity of fat present, it was found impossible to obtain microphotographs of the normal thin sections. In very thick (20 μ) sections, however, the thickness enhanced the staining effect, and in addition to the vesicular membrane, the nerve cord and the central excretory vessels stain lightly (Pl. 8, fig. 7).

(b) Cultured larvae. After cultivation in all media mentioned above (Section (1)), larvae were found to contain very large quantities of fat (Pl. 8, fig. 6). Indeed, so much fat was present that it is difficult to define its exact distribution. The main quantity was present in the parenchyma and the longitudinal muscles, but under oil immersion fine droplets were found to be present over the entire section with the exception of the central excretory canals. The lateral excretory canals were for the most part fat-free (they are not clearly visible in the microphotograph); some few contained small droplets.

(3) Distribution of glycogen

Glycogen was demonstrated by means of Best’s carmine or Langerhan’s iodine techniques, staining being effected by the modifications described in detail by Bensley (1939). With suitable fixation and imbedding both methods gave excellent results. For microphotographs sections were stained in the iodine solution without dissolving the wax; by this procedure the brown-red colour of the stained glycogen appears almost black against the white colour of the wax (Pl. 8, fig. 4) and can be photographed. Stained glycogen does not photograph well in slides cleared in the usual manner.

Material for glycogen demonstration was fixed in Carnoy and imbedded overnight in wax after careful dehydration and clearing. Preliminary trials had shown that this prolonged imbedding was essential for uniform results. In the preliminary tests, when a number of pieces of a fresh plerocercoid were fixed and imbedded in wax for the usual times (about 2 hr.), it was found that although some blocks gave sections rich in glycogen, others gave sections very poor in glycogen—although all the material used was from the same worm! When these irregular results were investigated in detail, it was found that after prolonged imbedding, uniformly stained sections of fresh plerocercoids were always obtained. Moreover, material
that had been imbedded for a short time, and found to give sections poor in glycogen, when *reimbedded overnight* gave sections rich in glycogen. It would appear therefore that the dense masses of intercellular glycogen found in cestodes are very impermeable to wax; and when not properly permeated the glycogen is easily lost in the staining solutions. When properly imbedded overnight, sections can be left standing in water for periods up to about an hour without any appreciable glycogen loss; with such sections it is unnecessary to use the usual celloidin film method.

(a) *Fresh uncultured larvae.* Considerable quantities present. Distribution confined mainly to two regions—the parenchyma and the outer periphery of the longitudinal muscles (Pl. 8, fig. 4). In these regions it is present in the form of large amorphous masses which almost completely fill all the intercellular spaces. Under high power it can be seen that glycogen is present as minute granules in the region of the circular muscle fibres, and in the subcuticula.

(b) *Cultured larvae.* It is difficult to compare the quantity of glycogen present after cultivation with that in fresh uncultured larvae owing to the fact that fresh larvae are wide in transverse section, whereas cultured larvae—owing to the relaxation during cultivation—are very narrow in section. Comparisons have therefore to be made between sections of different area. This being the case, it is advisable merely to record that glycogen is present; except in cases where there has been a considerable glycogen loss a quantitative comparison is not possible.

The following cultured larvae were examined:

| (i) | Broth | 84 hr. |
| (ii) | Broth + 1% peptone | 9 days |
| (iii) | Broth + 0.5% glucose | 6 days |
| (iv) | \(\frac{1}{4}\) Locke + 0.5% glucose | 84 hr. |
| (v) | \(\frac{3}{4}\) Locke | 37, 64, 84 hr.; 5 days |

Sections of (i), (ii) and (iii) showed considerable quantities of glycogen still present in the *non-degenerating* tissue regions. The degenerating scolex region and the regions immediately behind this were quite free from glycogen. (iv) was completely glycogen-free. In (v) quantities of glycogen were present after 37 and 64 hr. cultivation, but completely absent after 84 hr. and 5 days.

**DISCUSSION**

These experiments can be regarded as successful as far as the attainment of aseptic cultures is concerned, but unsuccessful as regards further growth and development of the plerocercoid beyond the primitive larval condition. The technique of aseptic cultivation has now been applied to the whole size range of plerocercoids: the largest known larval form, *Ligula intestinalis*, with a maximum size of about \(\frac{1}{2}\) mm.; an intermediate sized form, *Schistocephalus solidus*, with a maximum size of about 6 cm.; and the minute larvae—with a size range as given above—used in the present experiments. Since a great many pseudophyllidean cestodes have their plerocercoid phase in the coelomic cavity of fish, it seems likely that in time the aseptic cultivation technique will be successfully used on a number of these forms. The
Studies on tapeworm physiology

fact that location in the coelomic cavity of fish determines that larvae are living in an aseptic environment does not seem to have been generally realized.

With reference to the behaviour during cultivation, the strong attachment of the scolex to the wall of the culture tube gives some indication of the powerful muscular action of the bothria. The degree of development of the bothria in cestodes can thus be correlated approximately with the duration of life within the definitive host. The form of scolex in the larvae used in the present experiments is very similar to that of Diphyllobothrium latum and it is reasonable to conclude that in this latter form the scolex must become firmly attached to the intestinal wall of the host. Once attached, the cestode could undergo considerable growth, and in the case of D. latum there is a record of its persistence in man for a period of 8 years (Leiper, 1936).

In both Schistocephalus and Ligula, on the other hand, the bothria are very feebly developed—they are little more than shallow grooves. Attachment of these forms to the culture tube in vitro has never been observed, from which it is reasonable to conclude that a firm hold is never obtained on the gut wall in vivo. It can be speculated then, that these worms can only remain in the host gut for a short time. This is, in fact, exactly the condition found—the worms pass through the bird gut within 48 hr. (Schistocephalus) and 72–96 hr. (Ligula). The longer period of the latter is probably accounted for by the fact that the larger size of worm would find its passage down a coiled gut more difficult.

These few preliminary experiments, carried out as they were with a limited range of media, provide little additional information as to the nutritional requirements of cestodes. The available evidence, reviewed elsewhere (Smyth, 1947b) suggests that glucose, vitamins, hormones and an unidentified factor present in autoclaved yeast are necessary for normal growth and development. In the present experiments the variation between the normal behaviour periods between larvae in different media was statistically significant only in some cases.

Broth alone gave poor results, but the addition of 1% peptone was definitely beneficial. The further addition of glucose or yeast to the peptone-broth did not give significantly better results. The addition of 10% horse serum to the peptone-broth resulted in a marked improvement both in the normal behaviour period and the observable activity. This result suggests that the natural body fluids are likely to provide a culture medium more suitable for growth and development than one synthesized from simple inorganic and organic compounds.

The experiments with the saline solutions show that such solutions are useless for cultivation work, although results with these media are of interest. Behaviour was best in pure 1/4 Locke’s solution. The addition of 1 or 0.5% glucose was definitely detrimental to the normal behaviour. This result is interesting, for in the early experiments on Schistocephalus (Smyth, 1946) it was suspected that the addition of glucose to saline media had a detrimental effect on behaviour, although the results were not sufficiently uniform to allow a statistically significant result to be obtained. It is curious that glucose exerts such an effect on larvae in saline solutions, for the addition of 1% glucose to peptone-broth had no apparent effect—either beneficial or otherwise.
All the little available evidence on the carbohydrate metabolism suggests that glucose can readily be absorbed by cestodes and stored in the form of glycogen. Markov (1939) found that when the plerocercoids of *Diphyllobothrium latum* were kept in Locke's solution at 15°C for 38 hr., the glycogen content fell to 66% of its initial level; for the same period at 35°C the glycogen content fell to 22%. After 72 hr. at 35°C, the glycogen content fell to 6%. In Ringer-Locke + 1% glucose after 38 hr. cultivation at 35°C a gain of 3% in the glycogen content was recorded. These results are possibly open to criticism on the grounds that the experiments were not conducted under sterile conditions; for in non-sterile media, behaviour is very abnormal and degeneration rapidly sets in. Since glycogen is a notably labile substance and rapidly disappears from dead or degenerating tissue, the glycogen losses may have been due to this cause, although the fact that a gain was recorded in glucose-Locke after 38 hr. would suggest that the tissues remain normal for at least this period. However, the fact that non-sterile cultures were used introduces an uncertain factor into Markov's work, and his results—though of great interest—must be accepted with caution. Reid (1942) carried out feeding experiments on chickens and found that the glycogen content of the cestode *Raillietina cesticillus* fell to one-eleventh of its initial level after 20 hr. starvation of the chicken host.

In the present experiments, the results of histochemical analysis of glycogen carried out on larvae after cultivation in various media, while not at variance with Markov's results, are so inconclusive and uncertain that they provide little additional data on the carbohydrate metabolism.

The fact that in pure broth (i.e. a glucose-free medium) considerable quantities of glycogen are still present after 84 hr. cultivation suggests that either the cestode can synthesize glycogen from some of the complex substances* present in the meat extract or that the glycogen loss is not as considerable in this form as the results of Markov and Reid would seem to suggest. The low viability in pure broth did not allow glycogen determinations to be made after a more prolonged period. It is thus not possible to draw any conclusions from the presence of considerable quantities of glycogen in larvae after cultivation in broth + peptone (9 days), or broth + glucose (6 days). The cestode may be able to synthesize glycogen from peptone as well as from glucose, but there is no evidence that this is so.

Under starvation conditions, i.e. in ¼ Locke's solution, the complete disappearance of glycogen from larvae cultured for 84 hr. or longer would seem to support the hypothesis that the glycogen reserves in cestodes are very rapidly used up under starvation conditions. It is doubtful, however, whether such a conclusion is justifiable on this evidence alone, for considerable quantities of glycogen were present after 64 hr. cultivation, and it is difficult to believe that very little glycogen was used during the first 64 hr. of cultivation and that all the glycogen was suddenly consumed during the following 20 hr. A more likely explanation is that after about 72 hr. the worms were slowly dying and the disappearance of the glycogen was due to this cause. The results in ¼ Locke + 0.5% glucose would seem to confirm this

* Albumose, gelatine, peptones.
latter conclusion. If the larvae had been quite normal when fixed, and glucose was being absorbed, the tissues would have been rich in glycogen. Histochemical examination, however, revealed that all the glycogen had disappeared after 84 hr. cultivation. It seems wisest therefore to conclude that behaviour in salines or glucose-salines is abnormal and for this reason it is not justifiable to draw any conclusions as to the carbohydrate metabolism.

In contrast with glycogen, the increase in fat in sections of cultured larvae as compared with fresh larvae was so very striking and consistent that it can be concluded, with certainty, that fat is produced during cultivation. The fact that fat is a by-product of cestode metabolism has long been suspected but definite proof has been lacking; previous evidence has been summarized elsewhere (Smyth, 1947b). Brand (1933) suggested that the fat is formed probably by the anaerobic breakdown of glycogen. In both Ligula and Schistocephalus a similar production of fat has recently been demonstrated (Smyth, 1947c) and in these latter forms there is a marked excretion of fatty acids into the culture medium and the pH falls very rapidly. The fact that no pH drop was recorded in the present experiments is accountable when the very minute size of the larvae is considered. As pointed out already, the results from the histochemical glycogen investigations were very indefinite and it cannot be concluded that this fat is produced at the expense of the glycogen. Further investigations of the glycogen-fat relationship must await detailed chemical analysis.

The results of the present experiments have shown that a simple medium such as peptone-broth, in which large plerocercoids of Ligula and Schistocephalus (i.e. those containing genitalia anlagen) can become sexually mature, is not suitable for the growth and development of minute larvae. The problem of the in vitro cultivation of these small forms can therefore be considered to be in a position similar to that of animal and plant tissue culture after the early pioneer experiments had been carried out, namely, the basic technique of aseptic cultivation has been established, but a suitable medium in which growth and development will take place had not yet been elaborated. The elaboration of such a growth-producing medium must necessarily be a matter of empirical research. In addition to the composition of such a medium, physico-chemical factors—pH, osmotic pressure and temperature—must also be investigated in detail.

SUMMARY

1. Unidentified plerocercoids of the family Diphyllothriidae were removed aseptically from the coelomic cavity of Gasterosteus aculeatus and cultivated at 40°C in various liquid nutrient and saline media under sterile conditions.

2. The most successful results were obtained with peptone-broth + 10% horse serum in which larvae remained active and behaved normally for 10 days. Broth without peptone gave poor results (max. 2 days) but the addition of peptone (max. 7 days) greatly improved the activity. The addition of glucose or yeast to peptone-broth was not significantly beneficial. Survival times in all nutrient media were considerably longer than the periods of normal behaviour.
3. In 3/4 strength Locke's saline, the maximum period of normal behaviour was 4 days. The addition of glucose (0.25–1%) to this saline was detrimental to the behaviour of the larvae.

4. Histochemical investigation showed that fresh larvae were rich in glycogen but almost fat-free. The occurrence of glycogen in larvae after cultivation in various nutrient and non-nutrient media was such that no conclusions regarding the carbohydrate metabolism could be drawn. All larvae after cultivation, however, contained great quantities of intracellular fat, from which it was concluded that fat is a by-product of metabolism.

5. During cultivation in the more favourable media, larvae became firmly attached to the wall of the culture tube by means of their bothria; the strobila also became greatly elongated, and segmented in some cases. Histological examination failed to detect mitosis in the nuclei of the strobila and it was concluded that growth of the strobila had not taken place. The elongation of the strobila was presumed to be a result of the relaxation of the longitudinal musculature.

6. Of eight larvae kept in broth + 1% peptone + 0.5% glucose at room temperatures, seven still showed active undulation on exposure to light after 80 days' cultivation.

REFERENCES

EXPLANATION OF PLATE 8
Fig. 1. Enlargement of evaginated scolex shown in fig. 3. Gilson; borax carmine. × 110.
Fig. 2. Entire plerocercoid fixed immediately after removal from fish. Scolex invaginated. Gilson; borax carmine. × 30.
Fig. 3. Entire plerocercoid after relaxation in tap water. Scolex evaginated. Gilson; borax carmine. × 10.
Fig. 4. Transverse section of uncultured plerocercoid showing distribution of glycogen confined mainly to parenchyma and muscular regions. Section stained and photographed without removal of wax. Carnoy; Lugol's iodine. × 100. (This microphotograph has been considerably touched up; staining of glycogen is intense but does not photograph too well.)
Fig. 5. Transverse section of plerocercoid after cultivation for 5 days in peptone-broth. Note great enlargement of lateral excretory canals. Bouin. Haematoxylin and erythrosin.
Fig. 6. Transverse section of plerocercoid after cultivation for 9 days in peptone-broth. Great quantities of fat present. Formol-saline; gelatine imbedded; Sudan Black.
Fig. 7. Transverse section of fresh, uncultured plerocercoid; very thick section (20μ). Very little fat present, and only found in vesicular membrane (H), nerve cord (N) and central excretory canals (EC). Parenchyma and muscle layers fat-free. Compare with fig. 6. Formol-saline; gelatine imbedded; Sudan Black.

Abbreviations: E, lateral excretory canals; EC, central excretory canals; H, vesicular membrane enclosing larva; N, nerve cord; V, invaginated scolex.