THE DEVELOPMENT OF *DIPLOSTOMUM PHOXINI* (STRIGEIDA, TREMATODA) *IN VIVO* AND *IN VITRO*

THESIS

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INTRODUCTION

The axenic culture of parasitic helminths presents many problems, chief of which are: (1) the establishment and maintenance of aseptic conditions; (2) the development of a medium possessing physical properties (i.e. osmotic pressure, pH, temperature) simulating those of the host environment; (3) the provision of highly nutrient media, presented in a form capable of being utilized by the parasite and sufficient to satisfy the nutritional requirements of growth and maturation; (4) the provision of cultural conditions which will permit the rapid removal of metabolic wastes surrounding the developing helminth.

Although much work has been carried out on the axenic cultivation of parasitic cestodes and nematodes, investigations into the nutritional requirements and growth factors of parasitic trematodes have been few.

Most workers in the field of trematode cultivation have estimated the success of their experiments on survival grounds alone. Thus Yokogawa and others (1955) have recorded periods of survival up to 98 days with the metacercariae of Paragonimus westermanii, while Ross & Beuding (1950) and Senft & Weller (1956) have maintained alive the schistosomulae of Schistosoma mansoni for several weeks. More recently, Rohrbacher (1957), working with adult Fasciola hepatica, recorded survival periods of 21 - 30 days with these flukes.

The work of Ferguson (1940) is outstanding in that he succeeded in rearing the metacercariae of Posthodiplostomum minimum to the adult form; however the spermatozoa and eggs produced were abnormal.

The object of the work described in this thesis was to discover the physical and chemical conditions which stimulate the metacercarial stage of the trematode Diplostomum phoxini to mature in the intestine of the avian host. In order to do this, a series of experiments was carried out in which the metacercariae, after aseptic removal from the brains of minnows, were cultured under known conditions. As a result of these experiments, it has become possible to state that under certain conditions, the metacercariae of D. phoxini will mature in vitro. However, it is important to point out that although this gives an indication of the type of conditions which stimulate maturation of the fluke within the bird's intestine, it does not imply that the conditions of culture simulate those of the host environment but merely present an acceptable alternative.

EARLY DEVELOPMENT OF *DIPLOSTOMUM PHOXINI* IN VIVO AND IN VITRO

SECTION I

INTRODUCTION

Strigeid trematodes are in many ways particularly well suited for investigations into helminth physiology. The metacercariae which occur in sterile environments such as the peritoneal and pericardial cavities, are relatively easy to transfer aseptically into culture media. Another advantage of working with strigeid metacercariae is that those which occur in the eye or brain are not surrounded by a host cyst, and therefore the difficulty of freeing the larva from such tissue without damage does not arise. Although the metacercariae are lacking in genitalia, in other respects they are well developed, and maturation, with the development of genital organs, occurs very quickly after the gut of the definitive host is entered. This simplifies their *in vitro* cultivation, since their rapid development obviates the necessity of changing the medium and at the same time enables its value to be quickly assessed. Moreover, since the strigeids are small (usually less than 1 mm. in length), only a small volume of medium is required.

Nevertheless, despite these considerable advantages, little is known of the *in vitro* cultivation of strigeids, and consequently there is no information on their requirements of carbohydrates, lipids, proteins, salts or growth factors. It is true that Ferguson (1940) succeeded in rearing

metacercariae of Posthodiplostomum minimum to the adult form, but neither the sperm nor the ova produced were viable.

MATERIAL AND METHODS

In the experiments described in this thesis, the strigeid metacercariae used were obtained from the brains of minnows (Phoxinus phoxinus). The minnows were caught in baited traps. Those not required for immediate use were transferred to aquarium tanks containing constantly aerated tap water. The fish were fed on dried Daphnia and the water was changed weekly, usually the day following feeding. Using this procedure the minnows could be maintained alive for at least two months. The minnows were obtainable all the year round, thus a supply of experimental material was always available.

The infection of minnows with strigeids is widespread in Great Britain, and in minnows examined from five localities in the west of Scotland, an incidence of 100 per cent was recorded although the degree of infestation varied from 20 - 100 larvae per brain. It would appear that the infection is acquired at a very early age since larvae, 5 - 10 in number, have been recovered from minnows only 2 cm. in length (Frost, 1943). A description of the metacercaria under the name of Tetracotyle phoxini is given by Ashworth & Bannerman (1927), and of the adults, Diplostomum phoxini by Arvy & Buttner (1954), who, following the nomenclature of Hughes (1929), use the name Diplostomulum phoxini for the metacercarial stage. A more detailed description of the adult morphology is given by Rees (1955), who uses the name

Table VI. Results of cultivation of D. phoxini in Yolk-Albumen plus Tissue Extracts at 40°C

MEDIUM & CULTURE CONDITIONS	DEVELOPMENT OBSERVED AT VARIOUS INTERVALS						
		Male Genitalia			Female Genitalia		
	Hrs	17	40	65	88	65	88	112
Yolk-Albumen + Duck Embryo Extract Shaken	Stage	mitoses - >200, >200,170, 180	commas & rosettes	mature sperm in testes	active sperm in seminal vesicle	ciliated uterus; diazo -ve	vitelline reservoir; 2 flukes with 1 egg each; diazo -ve	5,7,8 & 9 days: active sperm; 6 - 9 eggs*/ fluke diazo -ve
	No.ex.	16	27	21	25	21	25	50
Yolk-Albumen + Schistocepha-:lus Extract Shaken	Stage	mitoses - 110,210,200, 140	> 200 mitoses per fluke	active sperm in seminal vesicle	active sperm	ciliated uterus	3 flukes with 1-2 each; vitelline reservoir diazo +ve (brown)	5 - 6 eggs per fluke diazo +ve (yellow-brown)
	No.ex.	20	40	25	25	25	25	25

*Ova clearly visible within eggs.

Diplostomum pelmatoides Dubois, 1932, for the adult form. On the advice of Dr Prudhoe, of the British Museum, Faust's nomenclature of Diplostomum phoxini (Faust, 1918) has been retained.

A difficulty with in vitro work is the selection of suitable standards for the measurement of development. A standard such as maximum survival time contributes little useful information since survival without development is abnormal. Development to complete sexual maturity is the ultimate aim, but is too exacting for early work, when the production of sperm or ova is improbable. With large helminths, chemical assays may be useful to demonstrate increase in protein, phosphorus, nucleic acids etc., but such techniques are difficult to apply to metacercariae which weigh less than 20 µgm. It was finally decided to employ the method recently devised by Smyth (1956a) for the observation of cell division in platyhelminths, and, by determining the normal rate of cell division during the development of the fluke in vivo, to establish a standard with which in vitro results could be compared.

In Vivo Procedure

To determine the rate of development of D. phoxini in vivo, three 14-day-old ducks (Anas boschas domestica) were each fed with 6 - 20 heads of infected minnows. (Young ducks only a few days old were used because of the ease with

which the flukes could be recovered from their intestines.) The ducks were killed at various intervals after feeding and the duodenum and small intestine examined in 5 cm. sections under warm saline. The flukes were picked out with a Pasteur pipette and examined for genital development and rate of cell division. For calculation of the rate of cell division the flukes were transferred into 0.01 per cent colchicine in Tyrode, the pH being within the limits 7.0 - 7.6 (determined by glass electrode). After 4 hours' incubation at 40°C (avian blood temperature), they were removed and stained in 1 per cent aceto-orcein for 5 - 10 minutes at room temperature. The timing of the staining is not critical and depends on the condition of the stain but prolonged staining for more than 2 hours should be avoided as the flukes become 'leathery' and do not squash well. Lightly squashed preparations of the flukes were examined for genitalia and spermatogenesis under x200 magnification. For examination of cell division, preparations were squashed more firmly to obtain thinner films, and the dividing cells were counted under the oil immersion lens (magnification x950). A yellow-green (factor 2) filter slightly improved definition and reduced glare from the red stain. In each experiment, about 20 aceto-orcein squashes were prepared, 10 - 12 of which were used for mitotic counts.

<u>In Vitro</u> Procedure

Preparation of media

A. Basic Salt Solutions

Locke (without bicarbonate):

	Grm.
Sodium chloride (NaCl)	9.00
Potassium chloride (KCl)	0.42
Calcium chloride ($CaCl_2$)	0.24

Dissolved in 1,000 ml. water (glass distilled) and the solution autoclaved.

Tyrode[+] (without glucose or bicarbonate):

	Grm.
Sodium chloride (NaCl)	8.00
Potassium chloride (KCl)	0.20
Calcium chloride ($CaCl_2$)	0.20
Magnesium chloride ($MgCl_2 . 6H_2O$)	0.10
Sodium dihydrogen phosphate ($NaH_2PO_4 . H_2O$)	0.05

Dissolved in 1,000 ml. water (glass distilled) and the solution autoclaved.

Earle's (with iron):

Solution (1) -

	Grm.
Sodium chloride (NaCl)	6.80
Potassium chloride (KCl)	0.40
Magnesium sulphate ($MgSO_4 . 7H_2O$)	0.20
Sodium dihydrogen phosphate ($NaH_2PO_4 . H_2O$)	0.14

Dissolved in 500 ml. water (glass distilled) and the solution autoclaved.

[+]Tyrode: throughout the course of the in vitro experiments, Tyrode's solution has been modified to contain one per cent glucose and not 0.1 per cent as in original (see Parker, 1950).

Solution (2) -

	Grm.
Calcium chloride ($CaCl_2$)	0.20
Ferric nitrate ($Fe(NO_3)_3.9H_2O$)	0.001

Dissolved in 500 ml. water (glass distilled) and the solution autoclaved. Solutions (1) and (2) are mixed when cool.

Hanks':

Solution (1) -

	Grm.
Sodium chloride (NaCl)	8.00
Potassium chloride (KCl)	0.40
Magnesium sulphate ($MgSO_4.7H_2O$)	0.20

Dissolved in 400 ml. water (glass distilled).

Solution (2) -

Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$)	0.06
Potassium dihydrogen phosphate (KH_2PO_4)	0.06

Dissolved in 400 ml. water (glass distilled).

Solution (3) -

Calcium chloride ($CaCl_2$)	0.14

Dissolved in 200 ml. water (glass distilled).
Solutions (1) and (3) are mixed, then solution (2) is added and the resultant Hanks' solution is autoclaved.

The pH of all the above basic salt solutions was adjusted to the required level (determined by glass electrode) by the addition of sterile isotonic (1.4 per cent) sodium

bicarbonate. In early experiments, phenol red was added to give a final concentration of 0.002 per cent, to enable an easy check to be made on day-to-day changes in pH. The changes were small, however, and in later experiments, phenol red was omitted.

B. Basic Salt Solutions Plus Glucose

To the above mentioned basic salt solutions, glucose was added to give a final concentration of 1 per cent. The glucose was made up in isotonic solution to the saline and after autoclaving, the appropriate quantity was added.

C. Chick Embryo Extract

Eggs

It was arranged that twenty-four fertile hen eggs of 10 or 11 days incubation were available.

Sterile glassware required

Thirty crystallising dishes covered with large Petri dish lids.....wrapped.

Twenty-four 10 ml. pipettes.....plugged in tubes.

Fifteen 20 ml. hypodermic syringes.....in cardboard containers.

Thirty 1oz. universal containers (screwtop).

Miscellaneous equipment required

Large enamel dish.

Rubber tube with plugged mouthpiece.

Iodine in absolute ethanol (1 gm. in 100 ml.)

Two inch Petri dish for iodine.

One paint brush.

One pair curved forceps.

One scalpel.

Procedure

1. One litre of sterile Tyrode was prepared.
2. The crystallising dishes were unwrapped and laid out in a row on the bench.
3. Instruments were boiled in a sterilizer, then removed and placed in a convenient position on a rack.
4. The surface of the pointed end of an egg was painted with alcoholic iodine and allowed to dry. A vertical lamp was used to hasten drying.
5. Using a scalpel, a cavity was chipped open on the surface of the egg sufficient to permit the embryo to be removed. This was usually facilitated by allowing the liquid contents to flow out into a crystallising dish.
6. The neck of the embryo was grasped with forceps and transferred to a clamped syringe from which the plunger had been removed. The plunger was replaced.
7. The instruments were resterilized and the process repeated i.e. the second embryo was transferred to the same syringe.
8. The two embryos were forced through the nozzle of the syringe into a clamped universal container. It was convenient not to continue to the next stage until all the embryos had been transferred to the containers.
9. Using a 10 ml. pipette, 10 ml. of Tyrode was transferred to each container. The containers were allowed to stand overnight at room temperature.
10. The containers were centrifuged the following day at 1944 R.C.F.+ for 15 minutes.
11. The supernatant liquid was transferred to a fresh container using a 10 ml. pipette. This process was repeated until all the extract had been transferred.

+Relative Centrifugal Force.

12. The containers were left at room temperature overnight, by which time, any bacterial contamination was shown by a cloudy appearance of the extract.

13. The containers were labelled and stored in a refrigerator.

Setting up of Cultures

For in vitro cultivation of the worms, the strigeid larvae were removed aseptically from the brain of the minnow, which was held in a clamp facing the operator. The head was sterilized with alcoholic iodine and when it was dry the skin was scraped off the roof of the cranium and the skull resterilized. The roof of the cranium was removed by a median cut to expose the brain, which was taken out and placed in sterile balanced saline in a covered Petri dish. Many of the larvae emerged when the brain was teased with needles; they were picked out by pipette and transferred to another Petri dish for washing, before being collected and placed in culture.

At first, larvae were cultured in all-glass 5 ml. and 20 ml. eccentric-nozzle hypodermic syringes. Each nozzle was sealed by insertion into a small block of polyvinyl chloride (previously sterilized in boiling water), excess of the test medium poured into the syringe and the metacercariae transferred into the medium by Pasteur pipette. The plunger was then inserted into the barrel of the syringe, the P.V.C. block temporarily removed, the excess medium and air bubbles expelled while holding the syringe with the nozzle uppermost

(to prevent loss of larvae) and the block quickly replaced. Finally the top of the barrel was smeared with vaseline to prevent air gaining access to the medium. Stringent bacteriological precautions were taken throughout all these procedures. Later experiments showed that these syringes gave no better results than test-tubes.

Survival of the flukes in vitro was determined by twice-daily examination of the cultures by means of the reflecting microscope arrangement described by Smyth (1946). For the determination of the degree of development, the flukes were removed from culture and treated in the same way as that described above for flukes recovered from the duck intestine.

RESULTS

Development In Vivo

Development of D. phoxini to full maturity in ducks takes 3 - 4 days, by which time 1 - 5 eggs are visible in the uterus.

Morphology. The most characteristic change which occurs in the fluke's external morphology is the protrusion and growth of the posterior lobe. In the metacercaria (Fig. 1) this lobe is very small (less than one-fifth of the total body length) and, since it is easily retracted into the body, is often invisible. On entry into the host the worms become active and the posterior lobe is pushed out to give the characteristic bilobed strigeid appearance (Fig. 2). The lobe rapidly increases in size, until by the end of 24 hours it forms one-third of the body length. The increase continues with the development of the genital organs (Fig. 3, Fig. 4), which are mostly situated in the posterior lobe.

Cytology. Aceto-orcein squashes show that cell division is stimulated within a few hours of the metacercaria's entry into the host (Table I). Following a period of rapid mitotic cell division in the posterior lobe (Fig. 5 (a), (c)) meiotic figures appear in 24 - 30 hours (Fig. 5 (b)). By 34 hours, groups of young spermatids can be seen, which are easily identifiable by their small haploid nuclei (Fig. 5 (d)) and by the fact that they occur in clusters of 32. In the course of

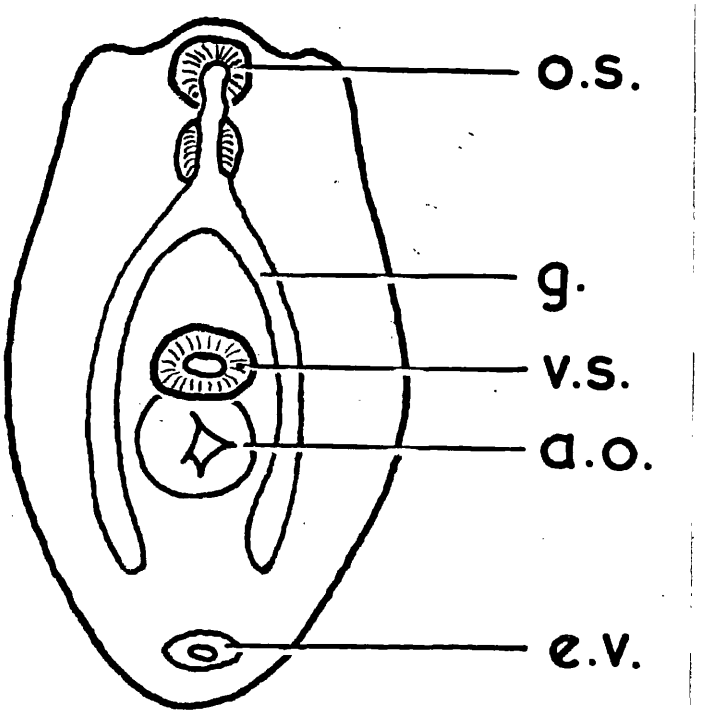

Fig. 1. Metacercarial stage of D.phoxini. (Posterior lobe withdrawn.)

a.o. adhesive organ; e.v. excretory vesicle; g. gut;
o.s. oral sucker; v.s. ventral sucker.

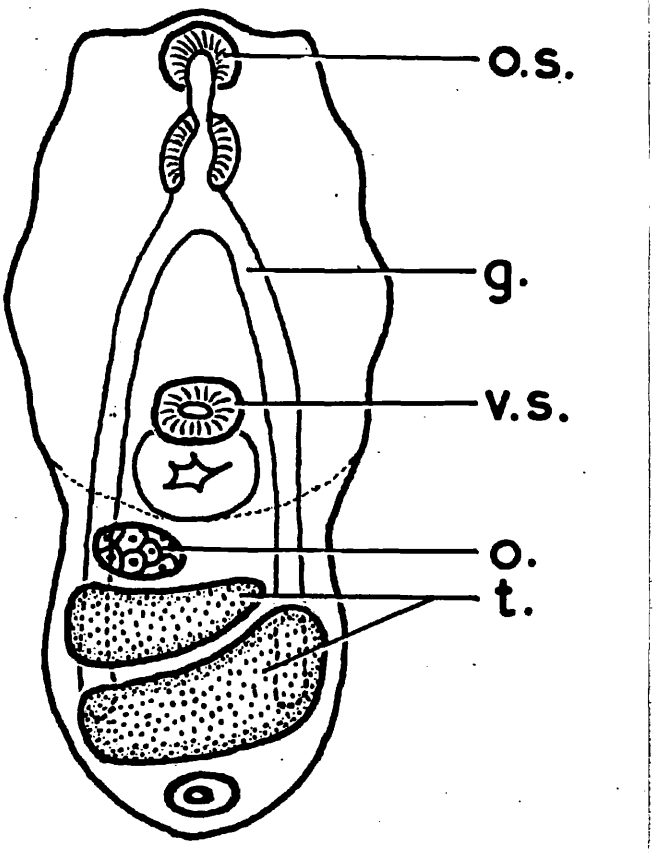

Fig 2. Bilobed adult of D.phoxini after 37 hours in the duck.

g. gut; o. ovary; o.s. oral sucker; t. testes;
v.s. ventral sucker.

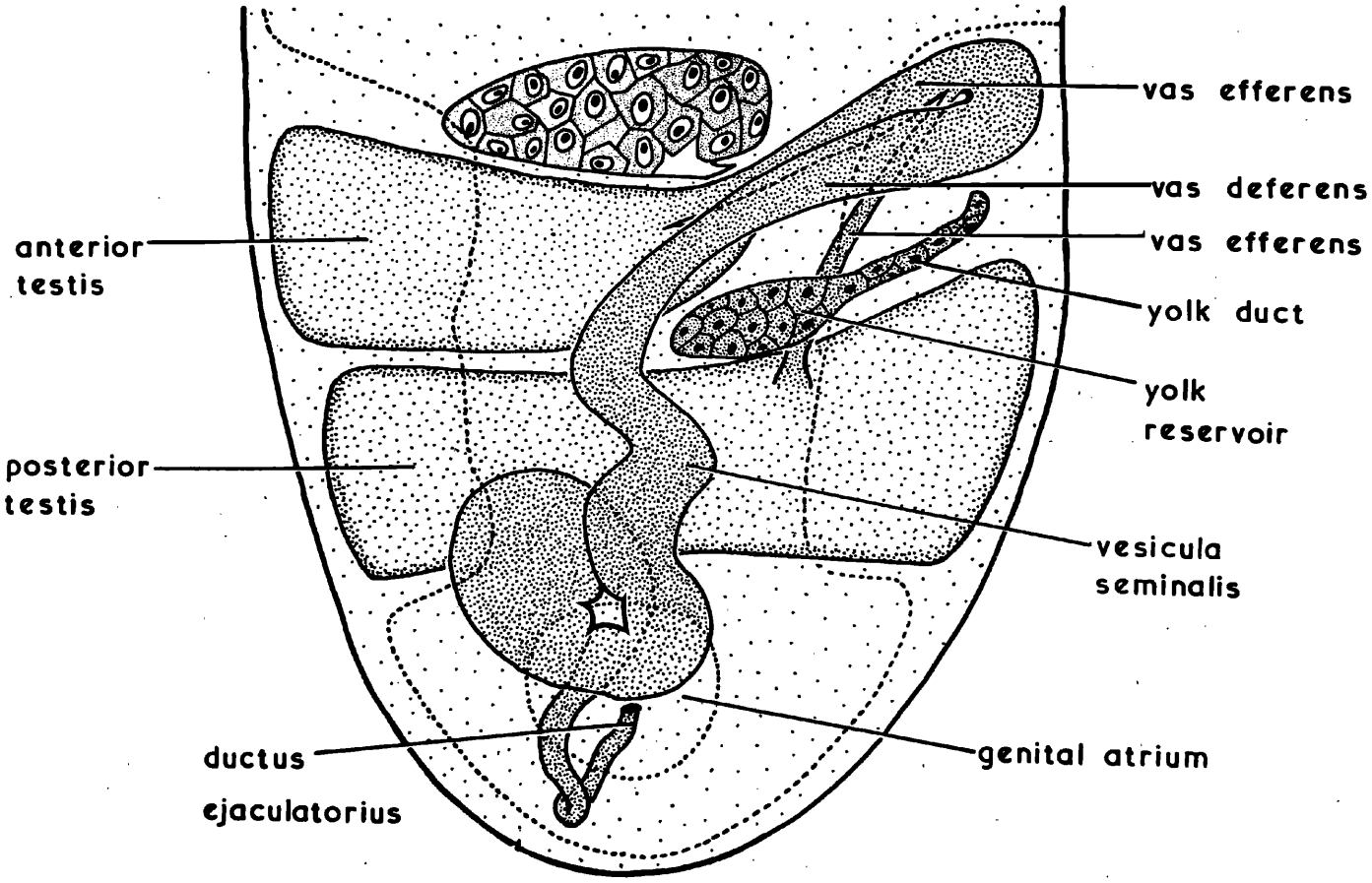

Fig. 3. Ventral view of male genitalia of D.phoxini. (After Rees).

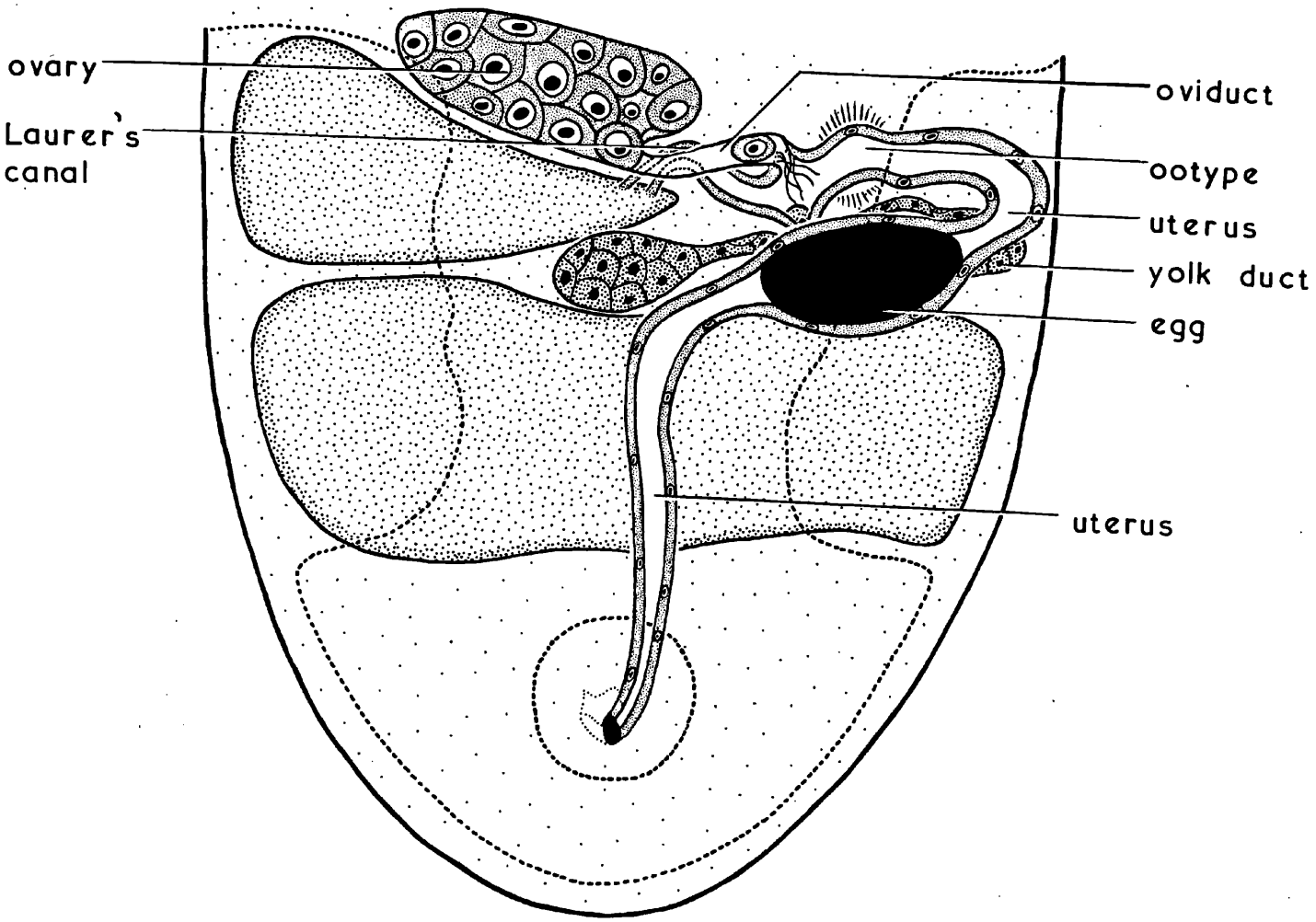

Fig. 4. Ventral view of female genitalia of D.phoxini. (After Rees).

Table I. Showing the rate of development of D. phoxini in young ducks, based on data obtained from the examination of at least 20 active flukes at each time interval.

No. of hours in duck	Average no. of metaphase plates found per fluke after an additional 4 hrs in colchicine
0	0 - 5 mitoses
6	more than 15 mitoses
18	" " 100 "
24	" " 250 "
	Later Development
28	meiosis; haploid nuclei
34	early spermatids present
36	'comma' spermatids present
40	spermatozoa present
72	occasional egg present
84	eggs present in uterus

the next few hours the small round spermatids elongate and become 'comma' shaped (Fig. 5 (e)), and by 40 hours, clusters of fully formed spermatozoa are visible (Fig. 5 (f)). By 72 hours (the next stage examined) eggs are sometimes present, but in Table I the time is recorded as 84 hours, since, by then, almost all the flukes have eggs in the uterus.

Development In Vitro

In an attempt to discover the conditions necessary for the flukes' maturation in vitro, metacercariae recovered from the brains of minnows were cultured in various media. The first media used, to determine whether the flukes required an exogenous energy substrate, were balanced saline solutions with or without glucose. The results obtained were as follows:

Balanced Salt Solutions (BSS). Four different salt solutions were tested at normal and three-quarters normal strength and at two pH levels - 6.5 and 7.5. They were: Locke; Tyrode (without glucose); Earle's saline plus iron; and Hanks' saline.

Ten to twenty metacercariae were placed in a hypodermic syringe and four syringes of each strength and of each salt solution were prepared. The cultures were incubated in the oven at 39°C. In all cases the larvae were dead within 18 hours. Replication with the fresh salines gave the same

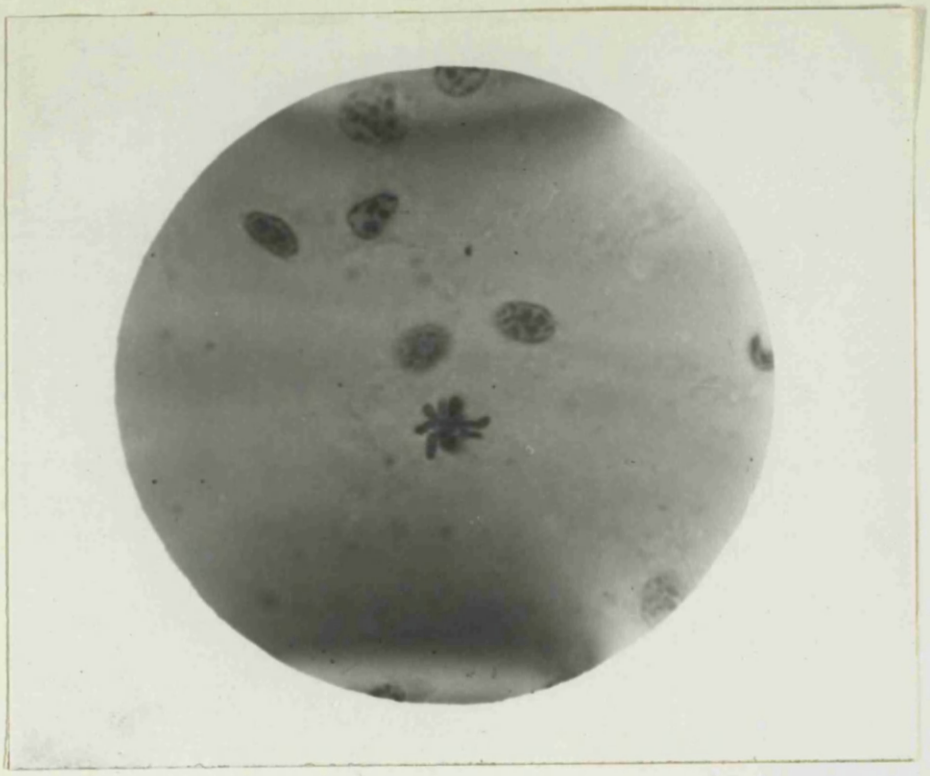

Fig. 5. (a) Prophase.

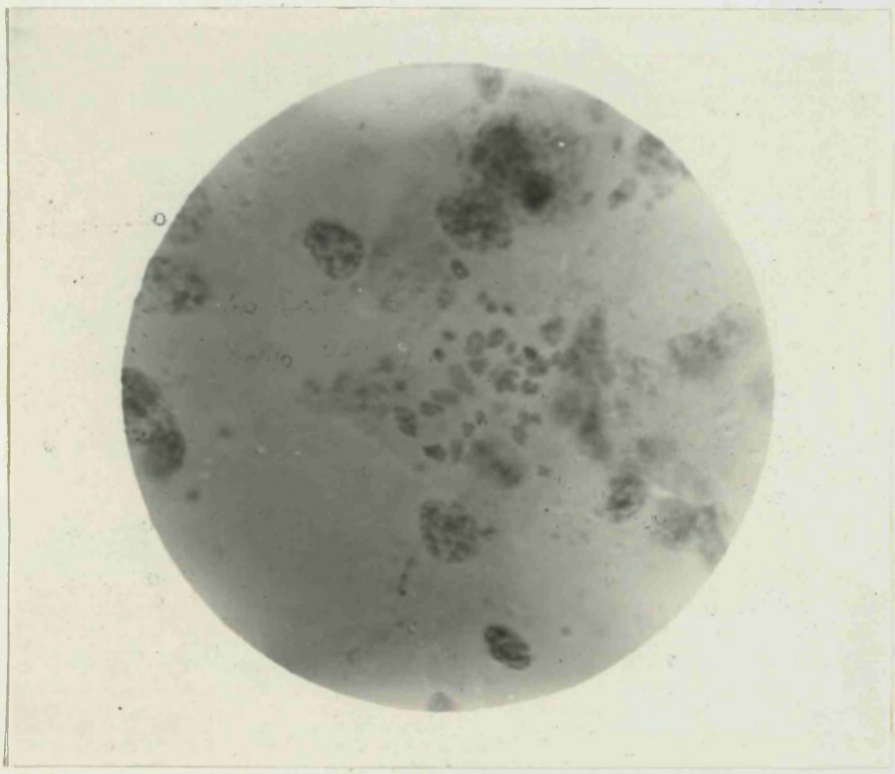

Fig. 5. (b) Metaphase of first meiotic division.

Fig. 5. Aceto-orcein preparations of D.phoxini, showing stages of spermatogenesis during development in the duck. (X 950).

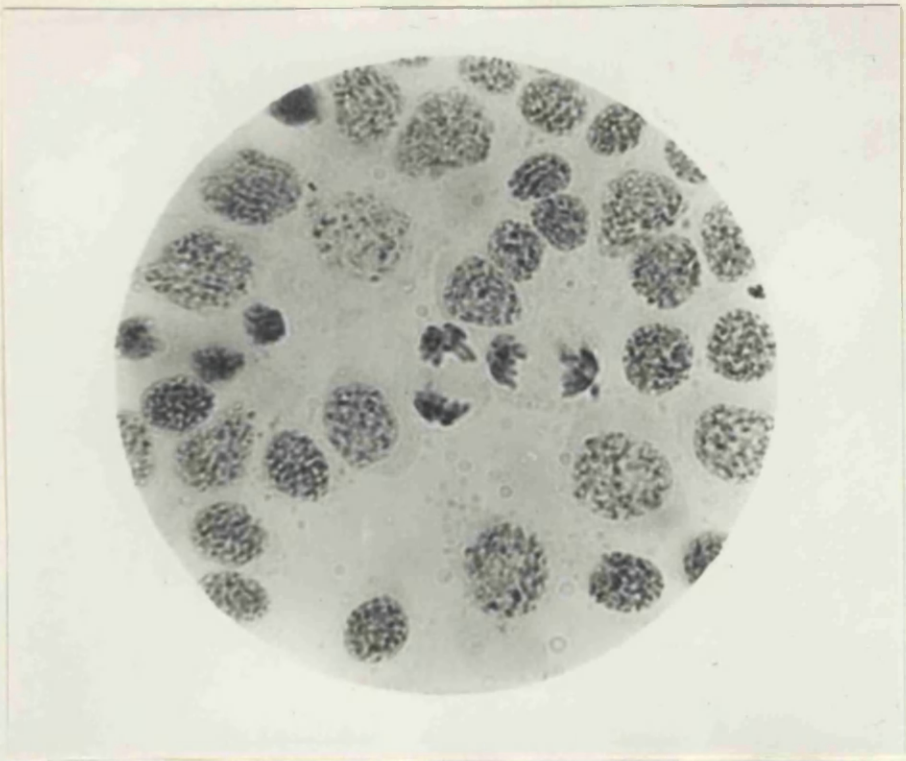

Fig. 5. (c) Anaphase.

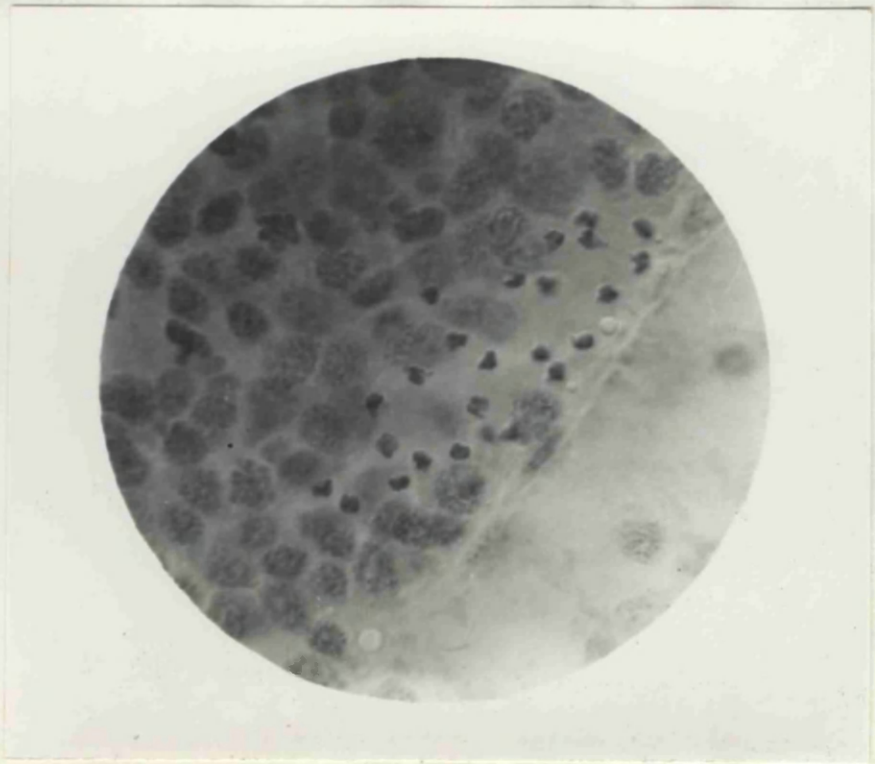

Fig. 5. (d) Early spermatids (after 36 hours).

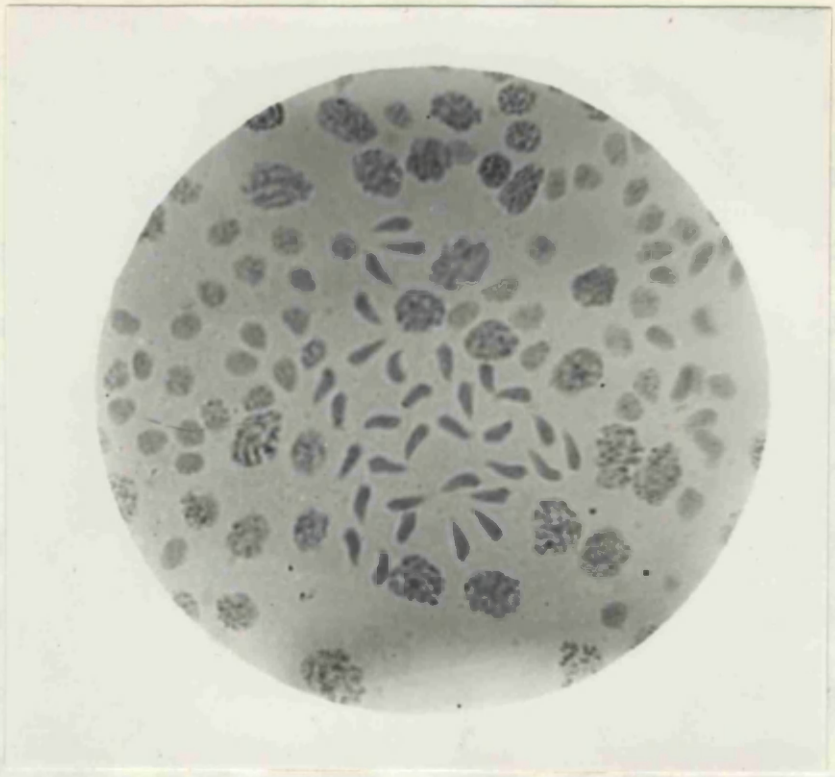

Fig. 5. (e) A cluster of 32 'comma' spermatids (after 36 hours).

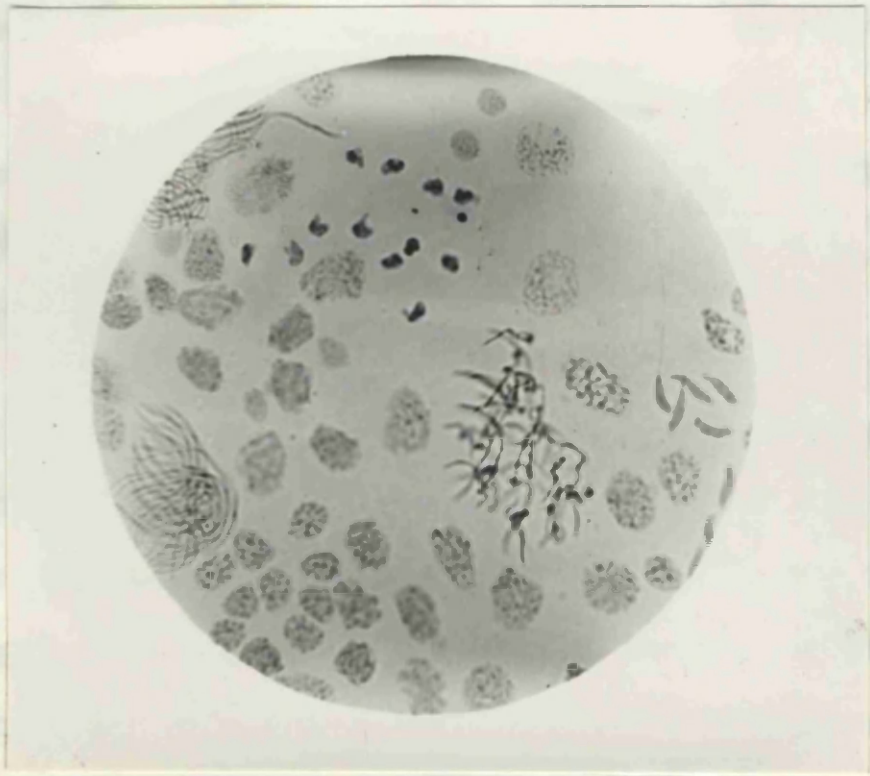

Fig. 5. (f) Sperm morula and spermatids (after 40 hours).

results (Table II).

BSS Plus Glucose. To half the saline used in the BSS experiments glucose was added to give a final concentration of one per cent. In all cases a great increase in longevity resulted; at least half the flukes survived for 72 hours, and in some cultures they were still active after 144 hours (Table II). Visual and microscopical examination showed, however, that no development of the posterior lobe had taken place. Replication with fresh glucose-salines gave the same results.

No difference in longevity was observed between normal and three-quarters normal glucose-salines; nor did the pH appear to be critical. The larvae in cultures begun at pH 6.5 and falling to 6.3 showed no difference in activity and survival from those in cultures begun at pH 7.5 and falling to 7.2 (pH determined by glass electrode).

Serum, Embryo Extract and Tyrode. In an attempt to supply the missing substance which stimulates maturation within three days in vivo, horse serum and chick-embryo extract were added to Tyrode. The serum was tested at two levels (45 per cent and 20 per cent), and the embryo extract which was prepared from 11-day-old chick embryo, was added at a 4 per cent level. Sixteen cultures were set up in hypodermic syringes, eight with 45 per cent serum and eight with 20 per cent; embryo extract was added to four of each.

Table II. Longevity of D. phoxini in Basic Salt Solutions (Glucose absent/present).

BSS	No Glucose Longevity -hrs	1% Glucose Present Longevity -days
Locke	less than 18	4 - 5
¾ Locke	" " 18	4 - 6
Tyrode	" " 18	4 - 6
¾ Tyrode	" " 18	5 - 6
Earle's	" " 18	4
¾ Earle's	" " 18	4
Hanks'	" " 18	5 - 6
¾ Hanks'	" " 18	5 - 6

The flukes in all but two of these were still alive and active when the cultures were removed after six days' incubation. In this respect serum was a better medium than glucose-saline, but microscopical examination failed to show any signs of genital development. No difference could be detected between worms cultured in 20 per cent or in 45 per cent serum, nor did the addition of embryo extract appear to have had any effect.

Serum, Embryo Extract, Glucose and Hanks' Saline. From the foregoing experiments it was apparent that, although the flukes could be kept alive in sterile media for a period much longer than was necessary for sexual maturation in vivo, the genitalia failed to develop. It was therefore decided to examine the flukes after 72 hours' cultivation to ascertain whether any of the following media were stimulating the nuclear activity which is characteristic of early development in vivo (Table I).

One or more of three substances (20 per cent horse serum, 4 per cent chick embryo extract, 1 per cent glucose) were added to Hanks' saline. The seven combinations and a control are shown in Table III, together with the nuclear division counts obtained from squash preparations. A factorial pattern was used to test for interaction between substances, as well as for the specific effects of each.

The results fall into three groups: 1, salines, in which

Table III. Showing the rate of cell division in D. phoxini following 72 hours' cultivation at 40°C in various media. The numbers of dividing cells are shown in brackets, each number being the total count in one fluke.

Hanks' saline (Control) (died on first day)	Hanks' saline + embryo extract (died on first day)	Hanks' saline + glucose (0,1,4,0,6,0,2,2)	Hanks' saline + embryo extract + glucose (1,13,5,3)
Hanks' saline + horse serum (32,14)	Hanks' saline + embryo extract + horse serum (2,6)	Hanks' saline + glucose + horse serum (14,27,1,13,26)	Hanks' saline + embryo extract + glucose + horse serum (14,40,12,6,36,32)

the larvae died; 2, glucose-salines, in which they lived but showed little sign of an increased rate of cell division; and 3, serum media, in which there was a definite stimulus to nuclear activity. The addition of embryo extract had no effect, but, in view of the extremely labile nature of such extracts, much more thorough investigation is indicated before any conclusions can be drawn. Replication with fresh media gave the same results.

The Rate of Cell Division in Worms Cultured in Glucose Serum. Flukes were removed from culture at 24-hour intervals to determine whether the rate of cell division observed at 72 hours in worms kept in glucose serum was a constant figure, or whether it was merely a chance figure situated on either an ascending or a descending slope of a curve. The number of dividing cells was counted in 6 - 8 flukes each day. Of the four highest, the means were 48 after 24 hours, 30 after 48 hours, 25 after 72 hours, 3 after 96 hours and 1 after 120 hours. (The reason for averaging only the four highest was that occasionally very low counts are obtained, for no apparent reason, in any medium (cf. Table III), and it was considered that an average of the highest counts would be of greater comparative significance than a mean of all the counts.)

DISCUSSION

From the results of the above experiments it appears that the normal course of development of Diplostomum phoxini in ducks consists of three overlapping stages, each of approximately 24 hours duration. During the first day there is great mitotic activity, as shown by cytological examina-:tion; on the second day the male genitalia mature, with the production of motile spermatozoa; and on the third day there is maturation of the female genitalia followed by oviposition. It is with the first of these stages - the period of rapid cell division - that this paper is chiefly concerned.

Mitosis in the cells of the posterior lobe begins almost immediately after the metacercaria has been ingested by the definitive host. There is no direct evidence of the nature of the cells which divide, but, following the two meiotic divisions early on the second day, morulae of 32 spermatids are present. It seems probable, therefore, that most of the mitoses are spermatogonia dividing to form clusters of eight primary spermatocytes, similar to the development which occurs in Fasciola hepatica (Sanderson, 1953). Some of the dividing cells may be the anlagen of the more slowly developing ovary and vitellaria, and possibly of the genital tracts. In the anterior region of the body, where no genitalia develop, there is no evidence of increase in mitotic activity. It may be concluded, therefore, that

transfer from the minnow's brain to the intestine of the duck not only stimulates activity, but also causes a fundamental switch over in metabolism from somatic growth to genital development.

This switch over in metabolism is comparable to the change which occurs in the pseudophyllidean cestode Schistocephalus solidus, in which somatic growth is confined to larval development in the coelom of fish and entry into the bird's intestine immediately stimulates maturation. This pattern of development is different to that found in most tapeworms and probably to that found in most flukes, where a period of somatic growth precedes sexual maturity in the definitive host.

As the switch in metabolism is very rapid in D. phoxini, it is unlikely to be due to the formation of specific hormones or metabolites. It would seem more probable that some general physical mechanism actuates the change over, as in Schistocephalus sp., where temperature is the stimulus. This hypothesis - that the stimulus for genital development is, not the presence of a host specific metabolite, but some general physical factor - is supported by the work of Berrie (1956), who has shown that D. phoxini will develop to full maturity in mammalian as well as in avian hosts.

If the stimulus is merely a rise in temperature or some other change in physical conditions, some development is to

be expected in any non-toxic medium. The degree to which such development will proceed will of course, vary greatly according to the adequacy of the medium.

Examination of flukes cultured in salines is valueless, as the worms die quickly owing to lack of an exogenous energy substrate. This very short survival of strigeid flukes in non-nutrient media contrasts sharply with the fate of most tapeworms in similar cultures, the most likely explanation for which may be the difference in glycogen reserves. According to Odlaug (1955), the glycogen reserves of six different species of trematodes (one of which was in the metacercarial stage) varied from 0.4 to 1.7 per cent dry weight, whereas four different species of cestodes examined by Hopkins (1950), Archer & Hopkins (1958), Archer (1958), Hopkins & Hutchison (1958), contained from 40 to 54 per cent. In strigeids, where the ratio of volume to surface area is considerably smaller than in the frog trematodes analysed by Odlaug, an even lower glycogen reserve may occur.

In glucose media the flukes survived well, but examination after 72 hours showed that mitoses were occurring only in the flukes which were cultured in media to which serum had been added. At first it was thought that serum contained specific metabolites which stimulated genital development. A more probable explanation, however, is that it contains the basic nitrogenous products necessary to the fluke if it is to maintain the rapid rate of protein and

nucleic acid synthesis which has been stimulated. The absence of nitrogenous substrate in glucose-salines would result in a cessation of nuclear activity as soon as the endogenous reserves were exhausted. It is not surprising therefore, that only a very low rate of cell division was found after 72 hours in flukes cultured in glucose media.

This interpretation of the results does not account for the failure of sperm and ova to form in serum media. The reason may be unsuitable physical conditions or the lack of certain nutrients, although these are not the only possible explanations. The progressive fall in the rate of cell division suggests that even in serum the flukes were not obtaining sufficient nutriment. If D. phoxini ingests only solid food, it is possible that in a liquid medium like serum, only those substances are used which can be absorbed through the cuticle.

The use of colchicine in the experiments raises several interesting points. Its main advantage is that the counting of nuclear divisions is made much quicker and more accurate. Instead of searching for a number of different configurations, some of which (e.g. early prophase) are difficult to identify with certainty, the examiner is concerned only with the identification of one stage, the metaphase plate. The use of colchicine may also be of value in comparisons of flukes from media which are

supporting only a low rate of cell division. In such cases direct counts may be too small for purposes of comparison, whereas, on the expanded scale, made possible by several hours' treatment in colchicine, significant differences may become apparent.

On the other hand, the use of colchicine has certain disadvantages. In the first place it is a cell poison, and, although in the case of a number of organisms a level has been found at which it can be added without injury to the cell, while at the same time blocking cell division, the level varies with different organisms, and careful investigation is necessary to determine the limits in different helminths. Secondly, there is the possibility that the metabolic rate of a fluke removed from the duck's intestine and placed in colchicine may change rapidly.

However, despite these disadvantages, the in vivo pattern obtained, as demonstrated by the colchicine method, has served as a useful criterion in distinguishing between growth and survival in D. phoxini.

SUMMARY

1. The development is described of the strigeid trematode Diplostomum phoxini in the domestic duck. Maturation takes 3 - 4 days.

2. A simple technique is given for preparing aseptic cultures of the metacercarial stage taken from the brain of minnows.

3. The results of cultivation in various media are compared, with longevity and rate of cell division used as criteria.

4. The findings are discussed, with particular reference to the nature of the stimulus which initiates maturation.

CYTOLOGICAL AND HISTOLOGICAL CRITERIA FOR EVALUATING DEVELOPMENT OF TREMATODES *IN VIVO* AND *IN VITRO*

SECTION II

INTRODUCTION

Improvements in techniques for *in vitro* cultivation of parasitic helminths, depend, for their evaluation, on criteria for the recognition of the degree of development taking place under a particular set of environmental conditions. Such criteria must clearly be related to the 'normal' pattern of development in a susceptible host, using the term 'normal' in an operational sense to denote a maturation which culminates in the production of eggs capable of completing the life cycle *in vivo*.

The most satisfactory criteria are those which are (a) precisely definable (e.g. the appearance of active spermatozoa), (b) readily recognizable, either by direct observation or by means of simple techniques, and (c) cover the whole range of the maturation processes. The last condition is especially important and criteria for very early stages must be established if the prolongation of unsuccessful experiments is to be avoided.

Only in the smaller species of nematodes, is it usually possible to use visual criteria for the phases of maturation on account of the relative transparency of the larval forms. In trematodes and cestodes, the opaque nature of the tissues makes direct observation of these phases impossible, and a detailed study of the tissues or organs at a cytological,

histological or histochemical level becomes necessary when the choice of suitable criteria is being considered.

Since the early stages of maturation are characterized by the occurrence of intense mitotic activity, it has been suggested that an estimate of this activity, as demonstrated by colchicine inhibition, could serve as a useful criterion for early helminth development (Smyth, 1956a) and in the previous section attempts have been described to assess the development of Diplostomum phoxini in vivo and in vitro using this method. The application of the colchicine technique is considered in further detail here.

For the later maturation stages, resulting in the production of gametes and the materials forming the egg-shell, and for the maintenance of the 'normal' condition, other criteria of a cytological or histochemical nature are necessary. The establishment and application of such criteria are also described in this section.

Most of the methods employed have been developed during attempts to cultivate the metacercaria of Diplostomum phoxini to sexual maturity in vitro. However, the basic techniques are essentially those of Smyth, to whom reference is made in the text. Some of these methods may find wider application to in vitro helminth work in general.

MATERIAL AND METHODS

Metacercariae of Diplostomum phoxini were obtained from the brains of minnows. Adult trematodes were obtained by feeding entire minnow heads to ducks and, by performing autopsies at suitable intervals, flukes in all stages of development could be obtained. For morphological studies from whole mounts, the flukes were fixed in 70 per cent ethanol and stained as indicated later in the text. A set of 'standards' showing the expected extent of in vivo development each day was therefore available.

For in vitro work, metacercariae were obtained aseptically as previously described (Section I) and trans-:ferred to suitable containers for culture; the media and conditions of culture are described in detail in Section III.

IN VIVO DEVELOPMENT

The processes taking place in the maturation of a metacercaria to an adult trematode are essentially concerned with the development of genitalia for the production of the egg. A detailed account of the pattern of development is given in Table I. For convenience, development has been divided into a number of phases, the transition between each phase being marked by a change over to different criteria. It is recognized that this development is a continual process in which the borderlines between some phases are not readily definable. The phases selected are as follows:

Phase 0. Infective metacercaria. Larvae show no traces of genital primordia (Fig. 1A).

Phase 1. Cell multiplication. The provision of a large number of cells for organogeny; characterized by intense mitotic activity.

Phase 2. Body shaping. The formation of an adult body outline.

Phase 3. Organogeny. The appearance of genital primordia and their transformation into organs recognizable by their shape or position (Fig. 1B).

Phase 4. Early gametogeny. The occurrence of the early stages in spermatogenesis, oogenesis and vitellogenesis.

Phase 5. Late gametogeny. Maturation of spermatozoa and ova (Fig. 1C).

Table I. The pattern of development of Diplostomum phoxini

TIME IN HOST - HOURS	TOTAL NO. OF FLUKES EXAMINED	TESTES	OVARY	UTERUS
0	20	-	-	-
12	24	outline visible	-	-
24	19	clearly visible; organogeny well advanced	-	-
37	17	clearly visible	outline visible	-
48 - 53	40	active sperm in seminal vesicle; mature sperm in testes	maturing ova present	ciliated ute visible
55	40	testes partly masked by a few vitelline cells; active sperm present	clearly visible; ova have large nuclei and nucleoli	ciliated; nc
60 - 65	50	active sperm surround partially tanned egg in uterus	mature ova present	mostly obscu by vitellari untanned to tanned eggs present
72	27	seminal vesicle packed with active sperm	mature ova present	only 15% of had eggs; th were lightly tanned
90 - 96	26	active sperm present	mature ova present	uterus maske vitellaria; lightly tann eggs present
120	40	active sperm present	mature ova present	fully tanned present in m flukes
168	38	active sperm present	mature ova present	fully tanned present in m flukes

Table I (continued). The pattern of development of Diplostomum pho

TIME IN HOST - HOURS	TOTAL NO. OF FLUKES EXAMINED	DEVELOPMENT OBSERVED AFTER TREATMENT WITH COLCHICINE FOR 4 HOURS
0	20	0 - 6 mitoses per metacercaria; genitalia absent
12	9	more than 100 mitoses per fluke
24	20	1. mitoses counts of >250 per fluke recorded; no 'comma observed 2. 10 - 15 cells per fluke - resembled fat globules with granular centres - ? early vitelline cells
37	11	30 - 50 mitoses per fluke together with sperm developmen ranging from 'comma' stage to mature inactive sperm in testes; (mature sperm observed in only 2 of the 11 preparations); many early vitelline cells
48 - 53	25	sperm in all stages of development; many mature sperm in testes; developing vitelline cells present
55	17	all showed without exception: many mature sperm and intermediate developmental stages; developing vitelline cells; developing ova - individual ova tend to be hexagonal in outline
60 - 65	28	all stages of spermatogenesis visible also maturing and mature ova and vitelline cells
72	20	all stages of spermatogenesis; maturing and mature ova and vitelline cells
90 - 96	23	all stages of spermatogenesis, however mature sperm predominate; fewer 'commas' and early sperm visible; maturing and mature ova and vitelline cells observed
120	15	development as at 90 - 96 hours
168	15	development as at 90 - 96 hours

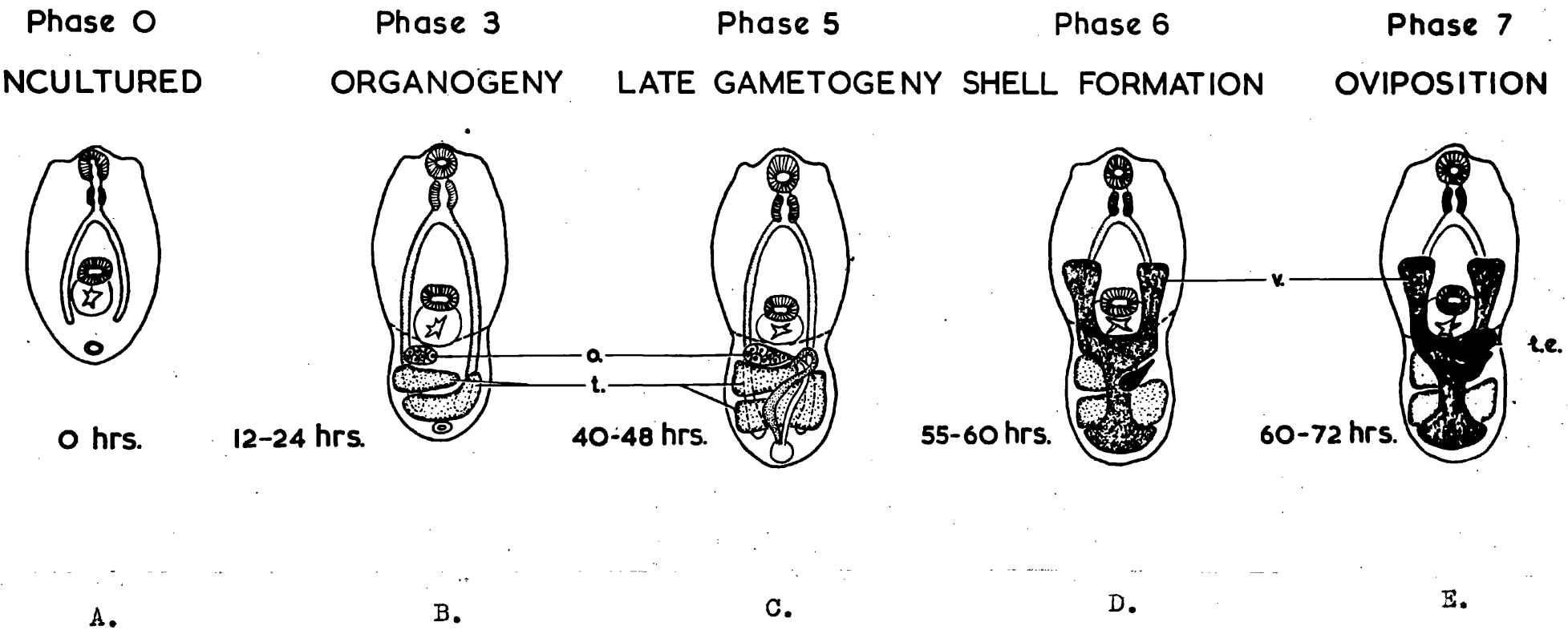

Fig. 1. Maturation of D.phoxini in vivo, showing the criteria used for the recognition of some of the phases.

o. ovary; t. testes; t.e. tanned egg; v. vitellaria.

(Note the ovary is shown in Figure 1B as it is assumed the ovarian cells are dividing during this period, but it is not visible until 37 hours as a discrete structure.)

Phase 6. Egg-shell formation and vitellogenesis. Appearance of egg-shell precursors and yolk (Fig. 1D).

Phase 7. Oviposition. The appearance of a fully formed shelled egg (Fig. 1E).

Phase 0. Infective metacercaria

In infective larvae, the genital tissues are undifferen-:tiated but the other structures appear fully formed (Fig. 1A). In aceto-orcein squashes, except for an occasional mitosis, nuclei show no activity. Development beyond this stage does not proceed until the definitive host is reached.

Phase 1. Cell multiplication

On ingestion by a warm blooded host, further development is initiated by the sudden rise in temperature and characterized by an outburst of intense mitotic activity. The mitoses may be conveniently detected by treating the larvae with colchicine, which causes inhibition at metaphase, and preparing aceto-orcein squashes (Smyth, 1956a). In Diplostomum the small size of the organism permits every mitosis to be counted, but in larger helminths in general, the size excludes this possibility and resource has to be made to methods of approximation (Smyth, 1956a).

Method. Details of the method have been given in the previous paper, but briefly it consists of removing the larvae from

the host or test medium and incubating in Tyrode's solution plus 0.01 per cent colchicine for 4 hours at 40°C. Following colchicine treatment the larvae are treated with aceto-orcein and squashes prepared. Squashes prepared in this way last for only a few hours unless the edges are sealed. The coloured cellulose preparation used for correcting duplicator stencils has been found excellent for this purpose and preparations so sealed are usable for 24 hours and often several days.

Results. In practice, this has been found to be one of the most useful criteria for evaluating the suitability of culture media and conditions. It has the advantage that an evaluation can be arrived at within 24 hours' cultivation, after which time, depending on the result of the mitosis count obtained, experiments may be abandoned or continued.

After 24 hours in the duck intestine, Diplostomum larvae each show more than 250 inhibited mitoses (Table II). This number was taken as the 'standard', and medium and culture conditions altered until activity of a similar order was achieved in vitro. Results from material cultured in vitro were so striking that only an approximate threshold figure was required. Thus in glucose salines, virtually no mitoses (0-6) occurred, although periods of survival up to 6 days are possible (Section I). In more recent experiments (Section III) using complex viscous media such as those containing egg yolk, counts of 50 - 100 mitoses were

Table II. Evaluation of culture media using mitosis as a criterion

MEDIUM	MITOSES after[1] 24 hrs culture	PROGNOSIS	MAXIMUM DEVELOPMENT ACHIEVED				
			TESTIS	OVARY	UTERUS	VITELLARIA	EGGS
Salines	0 - 6	poor	-	-	-	-	-
Yolk	50 - 100	fair	+	+	+	-	-
Yolk & albumen	> 200	excellent	+	+	+	+[2]	+[2]
Duck (control)	> 250	normal	+	+	+	+	+

1. Counts made after treatment with colchicine for four hours.
2. Developed abnormally.

obtained together with development up to late spermatogenesis. Further improvement of the medium by the addition of albumen, together with the alteration in the culture conditions by the introduction of discontinuous shaking to simulate intestinal movement, increased the mitosis figure to approximately 200 (Table II) and larvae cultured under those conditions ultimately produced active spermatozoa and eggs. Although the latter have proved to be abnormal (see p. 93), high initial counts, approaching those found in organisms matured in vivo, thus again served as excellent criteria for predicting successful early maturation stages.

Phase 2. Body shaping

The external changes which occur in trematodes are not so striking as in cestodes, where segmentation presents a useful though somewhat imprecise criterion.

In Diplostomum during the period 24 - 48 hours the oval outline of the body is replaced by the bilobed condition (Fig. 1B) typical of the adult strigeid; the posterior region, at first small, grows in size during subsequent maturation and the edges of the anterior region become foliaceous. This change in body shape, although recognizable by the experienced observer is not sufficiently precise to be used as a satisfactory criterion.

Phase 3. Organogeny

Organogeny, as a criterion, is in general more

satisfactory in cestodes than in trematodes. The detection of organ primordia can best be carried out in aceto-orcein whole mounts or well differentiated whole mounts prepared by any of the usual carmine or haematoxylin methods.

Early genital primordia in Diplostomum are not well defined due to the small size of the organism; their appearance does not become sufficiently definite until 48 hours *in vivo* by which time the presence of mature spermatozoa serves as a more easily recognizable criterion.

Phase 4. Early gametogeny

The detection of the various stages in spermatogenesis formed one of the most satisfactory of all the criteria used. These stages are readily seen in aceto-orcein squashes without previous treatment in colchicine.

The testes in Diplostomum occur as large discrete bodies. Early spermatogenesis is accompanied by intense mitotic activity and probably the majority of observed mitoses in Phase 1 occur in the testes. The 'rosette' stage is especially clear and together with the 'comma' stage form unmistakable criteria as to the progress of development.

Criteria have not been developed for the various stages of oogenesis. Maturing early ova give no characteristic histochemical reactions and in general present no criteria

for their early recognition.

The fully mature ovary of Diplostomum is recognized in squashes by its general form and position, individual ova having characteristically large nuclei and nucleoli.

Phase 5. Late gametogeny

This phase is characterized by the maturation and release of sexual products; the presence of mature spermatozoa serving as one of the most easily recognizable criteria of the whole developmental pattern. They may be detected in freshly teased worms or in aceto-orcein squashes. The mere presence of spermatozoa is not sufficient to indicate 'normal' development; spermatozoa must be morphologically normal, show movement and occur in quantities comparable to those occurring in worms matured in vivo. All combinations of abnormalities are possible: morphologically abnormal sperm morula showing no activity; morphologically normal spermatozoa showing no activity: morphologically normal spermatozoa showing activity but present in numbers less than those found in in vivo matured specimens.

The time taken for spermatozoa to appear, their quantity and activity have served as most useful criteria in assessing the development of Diplostomum. In early experiments, gradual improvement in culture conditions resulted in spermatozoa being produced in vitro after

66 hours' cultivation compared with 40 - 48 hours required *in vivo*; the quantity produced was smaller than *in vivo*. Further improvement in techniques resulted in a shortening of the maturation time to 40 hours and an increase in the numbers developed, until eventually no difference could be observed in the activity or the number of spermatozoa matured *in vivo* or *in vitro*.

Phase 6. Egg-shell formation and vitellogenesis

Mechanism of egg formation. As the male and female gametes near maturity, the rudiments of the vitellaria make their appearance as groups of cells which show at first no recognizable characteristics. As maturation proceeds, these cells increase in size and the precursors of the shell material appear in the cytoplasm as small globules. When the vitelline cells become fully mature, the egg-shell material is in the form of numerous large globules (Fig. 2) just visible in fresh preparations and which, in some helminths, have a yellow tinge. The mechanism of egg-formation (Fig. 2) follows the pattern typical of the majority of trematodes and pseudophyllidean cestodes (Dawes, 1940; Kouri & Nauss, 1938; Stephenson, 1947; Smyth, 1954b, 1956b; Gonnert, 1955). A fertilized ovum passes into the uterus region where it meets the 'vitelline' cells. Approximately at Mehlis' gland region, the vitelline cells release their shell globules which run together to form a semi-liquid shell which becomes moulded

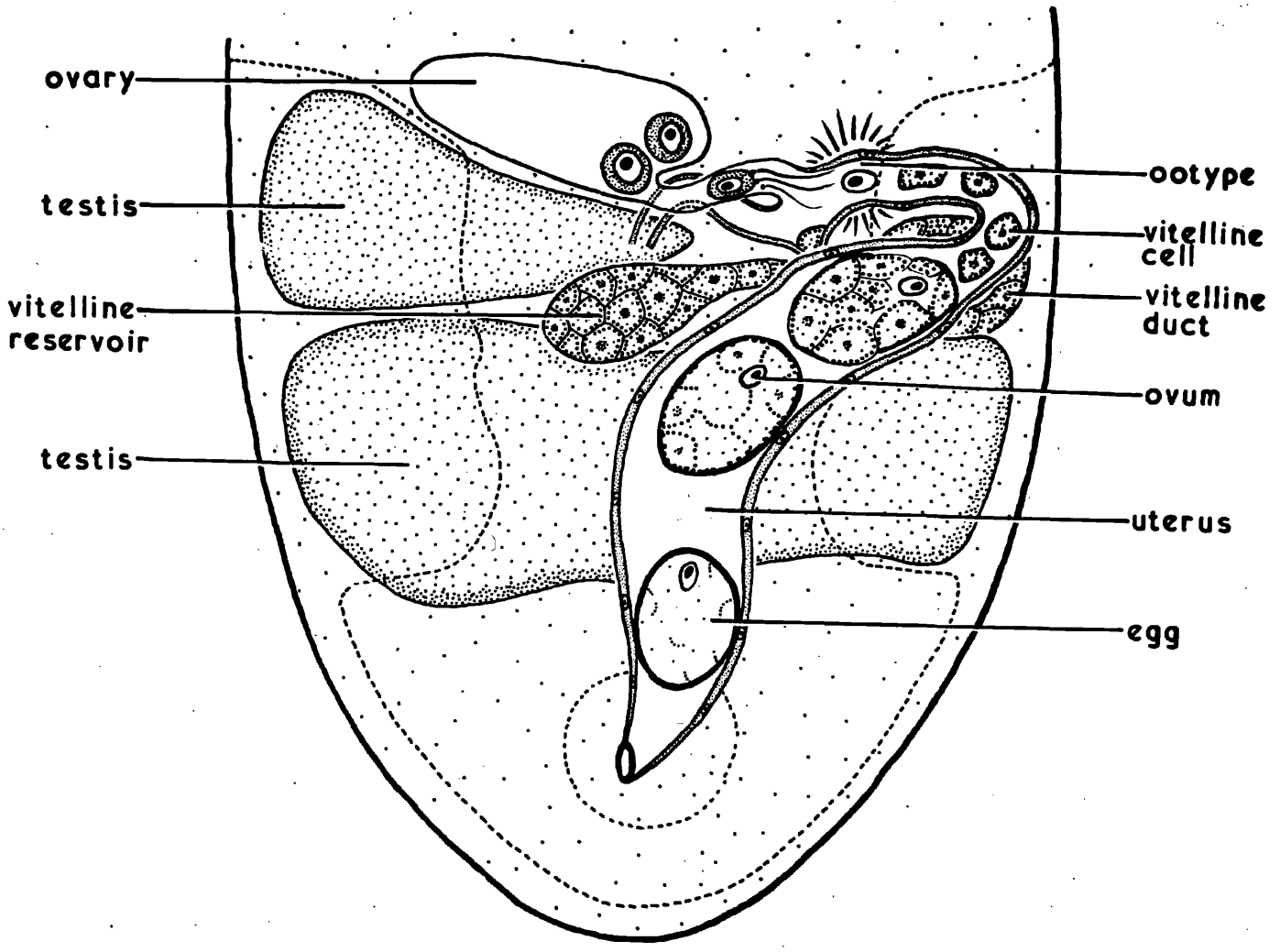

Fig. 2. Diagram of female genitalia of D.phoxini to illustrate method of egg-shell formation.

into an egg shape; the moulded egg passes down the uterus and its shell hardens and 'tans'. The remains of the vitelline cells serve as yolk for the developing egg. All recent work (Stephenson, 1947; Romanini, 1947; Vialli, 1950; Smyth, 1954b, 1956b; Gonnert, 1955; Johri & Smyth, 1956) suggests that the egg-shell in the majority of species in these groups is composed of sclerotin, a quinone tanned structural protein widespread in the animal kingdom, although several exceptions are known. The basic constituents of sclerotin are protein, phenolic substances and polyphenol oxidase, from which it follows that the egg-shell material in the vitelline cells may be traced by means of histochemical methods for detecting these substances.

Suitable histochemical methods for sections of whole mounts have been described in detail (Smyth, 1954b, 1956b; Johri & Smyth, 1956). The choice of methods will depend on the nature of the material and the reagents available.

Diazo technique for egg-shell precursors. One of the most useful histochemical techniques is that incorporating the use of stable diazotates which link readily with phenolic materials giving characteristic colours. The earliest appearance and subsequent fate of the egg-shell precursors may be detected by means of this method. It has the advantage that it may usually be applied to whole mount

preparations with consequent saving of time. Since the vitelline globules are small when they first appear, it is important that the specimens to which this test is applied should be flattened to the limit (without bursting) before and during fixation. 70 per cent ethanol, although poor as a cytological fixative, is the best general fixative for most of these tests which are concerned only with the presence of substances within a cell and not with the precise distribution of that substance within the cell. Details of the method have been given by Johri & Smyth (1956) but are repeated in outline below:

1. Flatten and fix in 70% ethanol 2 - 24 hours.
2. Wash in water - 15 minutes.
3. Place in 1% Fast Red salt B* (freshly prepared and filtered) in a watchglass and observe under a low power binocular. In small trematodes the phenolic shell precursors in the vitelline cells become red within ¼ - 3 minutes. Larger trematodes or thick cestodes may require up to 15 minutes.
4. Wash in water - 15 minutes.
5. Stain in Gower's carmine 1 - 3 hours (optional); differentiate in acid alcohol.

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*G.T. Gurr, London, S.W.6.

6. Dehydrate, clear and mount.

The colour developed in the egg-shell precursors depends on the nature of the particular phenolic compounds present. It ranges from yellow or orange to orange-red or bright red.

In Diplostomum specimens matured in vivo, the vitellaria become positive at 55 hours, some 5 hours before fully formed eggs appear (Table I). The colour reaction is orange-red to bright red, the vitellaria standing out brilliantly against an almost colourless background (Fig. 3A). In early attempts to mature these organisms in vitro, the most advanced stage of maturation obtained showed partly formed vitellaria, which in contrast (Fig. 3B) gave only a yellow or weakly orange-yellow diazo reaction.

This is strongly suggestive of an abnormal synthesis of the protein or phenolic precursors of the shell material possibly due to a nutritional deficiency in the medium. The nature of this deficiency has not yet been determined. The vitelline cells have many properties similar to those of other quinone tanning systems such as that secreting the byssus of Mytilus (Smyth, 1954b; Brown, 1952) or the egg capsule of elasmobranchs (Brown, 1955), and since the protein concerned in these systems is known from X-ray analysis to be collagen, it is possible that the protein of the trematode egg may likewise prove to belong to the

A. in vivo B. in vitro

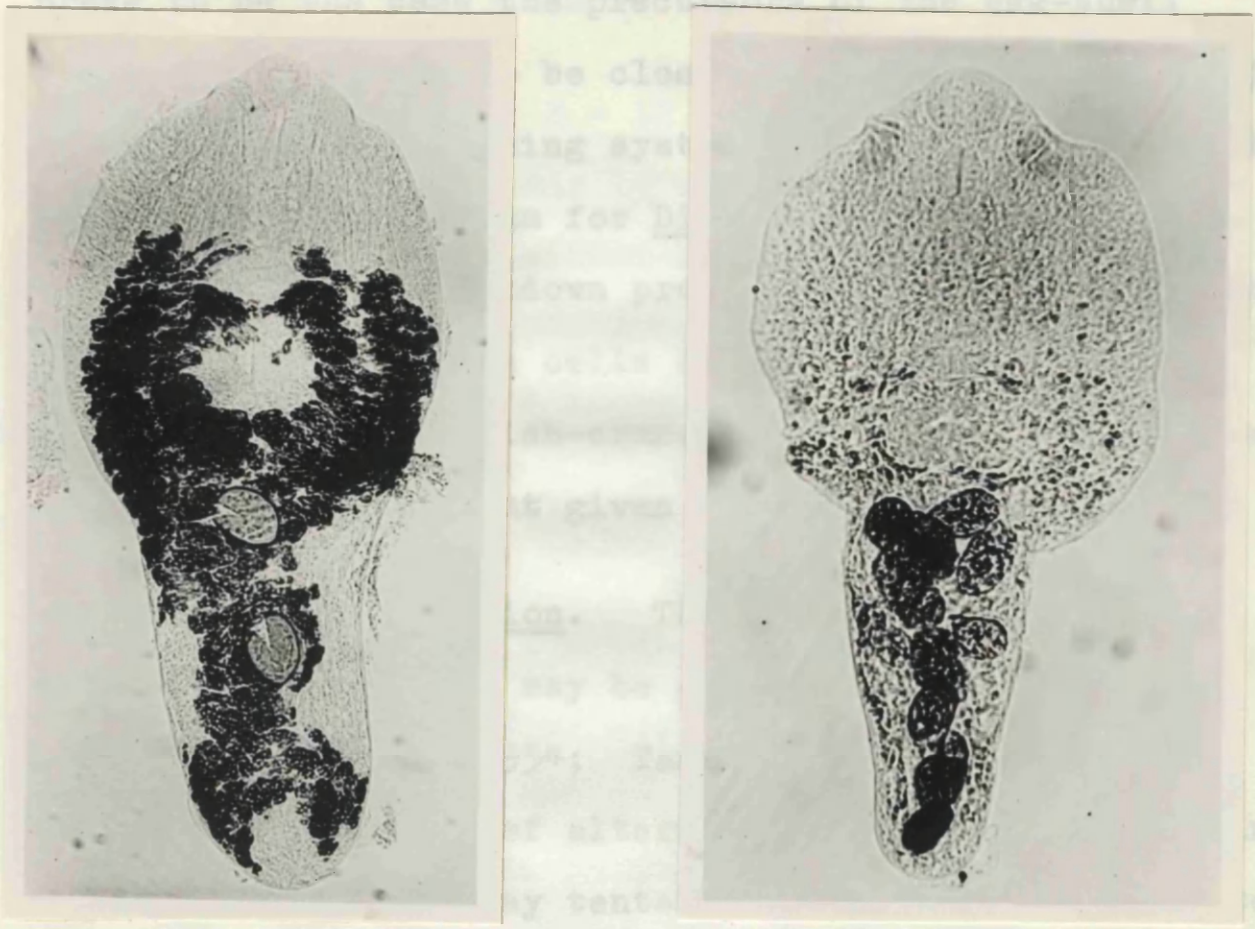

Fig. 3. Comparison of D.phoxini matured : A. in vivo; B. in vitro.

A. in vivo. in duck - 3 days.

B. in vitro. in yolk-albumen plus proline-hydroxyproline for 5 days. Fixation - 70% ethanol. Treated diazo reagent 3 minutes. Upgraded.
Note: Poorly developed outlines of the eggs and almost complete lack of diazo +ve material (egg-shell precursors) in the vitellaria of the in vitro specimen.

(X 100).

collagen group of structural proteins. If this should prove to be the case the precursors of the egg-shell material are likely to be closely related to those required in other collagen forming systems. It is suggestive that when the culture medium for Diplostomum is augmented by gelatine (i.e. a breakdown product of collagen - containing tissues) the vitelline cells although still poorly developed give a reddish-orange diazo colour reaction more nearly approaching that given by in vivo forms (Section III).

The argentaffin reaction. The argentaffin reaction is a non-specific one, and may be given by various reducing substances (Lillie, 1954; Tandler, 1955), but in the demonstrable absence of alternative substances in helminths, a positive reaction may tentatively be taken to indicate phenols, as only precursors of the shell material in the vitellaria have given a strongly positive reaction in all trematodes (and pseudophyllidean cestodes) studied (Smyth, 1954b, 1956b; Stephenson, 1947). Nevertheless, silver techniques are notoriously unreliable and results based on their use should be interpreted with caution. The method used in histochemistry is carried out in sections, but it may be adapted to whole mounts; vitellaria containing shell precursors react red to black depending on how long the reduction is permitted to proceed, and the concentration of silver nitrate used. In 5 per cent silver nitrate a positive result becomes evident in 1 hour; in 0.1 per cent

silver nitrate 12 hours are required. The technique for whole mounts is as follows:

1. Fix in 5% formol - 2 - 12 hours.
2. Wash in running water 15 minutes - 1 hour.
3. Place in 0.1% ammoniacal silver nitrate (add concentrated ammonia to 0.1% $AgNO_3$ drop by drop until the precipitate dissolves), 12 - 24 hours.
4. Wash in 1% ammonia - 2 hours.
5. Upgrade, clear and mount.

In Diplostomum the result closely parallels that with the diazo technique (Fig. 4), but the method is unsuitable for permanent whole-mount preparations as the cytoplasm may take on a reddish tinge on standing.

Alternative methods of detecting egg-shell precursors

There are a number of other methods of detecting egg-shell precursors, some of which may be used on whole mounts of helminths as well as sections, and serve as confirmatory evidence for the diazo reaction; others are too destructive or give colours cytologically unsatisfactory. The most useful of these methods is the catechol reaction for detecting polyphenol oxidase. This consists of incubating ethanol-fixed material in 0.1 per cent catechol at 40°C, a yellow to orange-red colouration developing at the enzyme site.

Fig. 4. Argentaffin reaction in D.phoxini after 60 hours in the duck. Note the result closely parallels that with diazo technique. (X 100).

With trematodes such as Diplostomum, the reddish colour developed is nearly as brilliant as with the diazo technique. Full technical details of the method are given by Smyth (1954b) and Johri & Smyth (1956).

This technique has a particular advantage over the diazo technique in that it not only gives a colour reaction with vitellaria but also with the completed egg-shell. Methods based on the presence of phenolic materials (e.g. diazo, argentaffin) gave either negative or only weakly positive reactions with the finished egg-shells situated far down the uterus as in this region the enzymatic oxidation of phenols to quinones has taken place, although the shells just formed may give quite strong reactions. A residue of polyphenol oxidase, however, remains in the shell so that the formed eggs give a characteristic catechol reaction. This method has the advantage of demonstrating the position and shape of the eggs in the uterus and is of value for the detection of abnormal uterine development.

Phase 7. Oviposition

General morphology. The oviposition phase is characterized by the appearance of the fully formed eggs. If numerous, these may be detected in the living material by gently compressing the helminths under a cover-glass. The colour of the egg-shell, its contours and size all serve as useful criteria when eggs of specimens matured in vivo and in vitro

are compared. A more precise evaluation may, however, be obtained by fixing the compressed specimens in 70 per cent ethanol and treating with catechol or diazo reagents, as in the previous section, when the eggs will become clear. In specimens of Diplostomum matured in vitro, only abnormally shaped eggs giving poor catechol and diazo reactions have been produced.

Testing egg embryonation. The final test of successful maturation, however, must be the fertility of the eggs and their subsequent development through the larval stages. For example, eggs from such forms as Diphyllobothrium latum or Fasciola hepatica matured in vivo show a range of 80 - 90 per cent embryonation. It is relatively easy to test embryonation of these species on account of the large number of eggs available. With small trematodes - e.g. certain strigeids - the number of eggs present in the uterus at any one time may only be one to five and the problem of testing embryonation without undue risk of loss or damage is more difficult.

A method found suitable for both small and large numbers of eggs is to place these on a cellophane disc (about 1½" diam.) in a watchglass covered with about ⅛" water. The watchglass is then placed in a Petri dish, and incubated at 26°C. After 24 hours at this temperature, the eggs become attached to the surface of the cellophane;

this prevents their loss when the water is being renewed daily and also greatly facilitates transfer of the embryonated eggs for subsequent hatching and infection of the intermediate host as the entire cellophane disc may be added to the host culture.

Eggs obtained from specimens of Diplostomum matured in vivo have been successfully embryonated using this method, the miracidia hatching out after 12 days' incubation at 26°C.

Criteria for early larval stages. In the present in vitro experiments, fertile eggs have not been obtained from Diplostomum, but some previous results with Schistocephalus and Ligula are relevant here. In Schistocephalus the earliest experiments resulted in abnormal eggs showing no embryonation (Smyth, 1946). Environmental conditions were later (1950) improved to permit the production of morphologically and cytologically normal eggs and although such eggs embryonated, the coracidia produced were under-:sized, unhatchable and believed to be haploid in genetic constitution due to failure of fertilization. This was confirmed when culture conditions were further improved by the introduction of the cellulose tube technique (Smyth, 1954a) to simulate the compression of the intestinal surfaces and assure fertilization; under these conditions, morphologically normal, full-sized hatchable coracidia were

then obtained.

Thus caution is clearly necessary even in the use of embryonation as a criterion and differences in size, inability to hatch or other abnormalities should be carefully watched for.

Criteria for maintenance of the adult condition

Once the fully mature condition is reached, it is important to develop criteria to determine whether or not the metabolism is being maintained at the adult level. This is similarly true when adult worms are initially used in culture experiments.

A continual supply of eggs equivalent in number and fertility to those produced *in vivo* would, of course, supply a precise criterion. One difficulty which arises here, is that although the total number of eggs may be immediately calculated, testing of fertility (by embryonation) takes at least several days at 26°C, so that immediate evaluation of the condition of the eggs is not possible. Other criteria of a cytological or histochemical nature suffer from a further disadvantage in that the cultured specimens must be destroyed before an evaluation can be arrived at. Notwithstanding these disadvantages, however, fertility and the absence of cytological abnormalities are the most satisfactory criteria at present available.

Cytological methods may give rapid evaluation. Work

on Schistocephalus (Smyth, 1946, 1952), Ligula (Smyth, 1949) and Fasciola (Clegg, 1957) has shown that the testes are extremely sensitive to unfavourable culture conditions, (similar observations have also been made while culturing Diplostomum). In Schistocephalus an unfavourable pH or temperature of cultivation is reflected in the appearance in the testes of 'giant' cells - abnormal spermatocyte or spermatic morula. These abnormalities may be detected in sections and often in aceto-orcein squashes although they show a tendency to burst with the latter technique.

In Fasciola, Clegg (1957) has shown that some cytological abnormalities can be detected after only 3 hours' cultivation in non-nutrient Hédon-Fleig, and that there is a steady increase in the percentage abnormality with time. In order to avoid tedious sectioning of every specimen, this worker has developed a technique of teasing the testes in saline, centrifuging and making smears. He has found that both the appearance of 'giant' cells and the relative number of morula showing metaphases can serve as criteria.

A combination of cytological examination and testing of egg number and fertility should provide a reasonably clear indication as to whether or not the metabolic activities of an organism are being maintained at the 'normal' adult level. This is especially important in biochemical metabolic studies in which data of a fundamental nature are being determined.

DISCUSSION

It has been shown that the development of the trematode Diplostomum phoxini presents certain features which may serve as criteria for assessing maturation in vivo. Early workers have used survival as an initial criterion for evaluating the success of helminth culture methods, but it is becoming increasingly clear that survival as a criterion, unrelated to the organism, is of little value except at the very onset of in vitro work. Its use may, in fact, produce grossly misleading pictures. Thus the cestode Schistocephalus matures in vivo in 36 hours and maturation is obtained in vitro in the same time under suitable conditions (Smyth, 1954a). After 36 hours' cultivation a worm produces quantities of eggs and continues to do so for 2 - 4 days, at the end of which time, having used up its endogenous reserves, it dies. On the other hand, if larvae are cultured in a small quantity of poorly buffered medium the acid excretory products released so inhibit metabolism that although the worms continue to show activity, little tissue differentiation takes place. 'Survival' under these unsuitable conditions may be as long as 10 days - more than twice its 'normal' longevity in vivo (Hopkins & Smyth, 1951). Similar observations were made by Ferguson (1940) during his attempts to cultivate to sexual maturity the trematode Posthodiplostomum minimum.

Thus, increased survival is not per se indicative of

improved *in vitro* conditions and may, as with *Schistocephalus* and *Posthodiplostomum*, point to exactly the opposite. The same pattern is followed in *Diplostomum* the larvae of which may survive and show activity for 6 - 7 days (Section I) in certain media without undergoing any further development. Application of precise criteria would eliminate such media as unsuitable after 24 hours' incubation. That survival is of little value as a criterion is still not yet generally appreciated by some workers, and Rohrbacher (1957) has recently estimated the success of *in vitro* experiments with *Fasciola* on survival grounds alone. With this organism Clegg (1957) has shown that cytological abnormalities rapidly develop in unsuitable media.

Criteria selected here for assessing development have been essentially cell division, spermatogenesis, egg-shell formation and fertility. These represent one set of criteria which have been found convenient, but alternative or additional criteria may emerge in subsequent work. A satisfactory criterion should be precise and readily and rapidly identifiable. The more precise and exact it is the more readily can slight changes in *in vitro* development be detected. It is important to detect a change in the developmental pattern as soon as possible, so that prolonged periods of cultivation should not be necessary before the growth producing powers of a medium are assessed. Used

singly or in combination, the criteria cited here can usually detect beneficial or detrimental changes within 24 - 48 hours at 40°C. Detection of mitosis after colchicine inhibition has proved the most efficient in this respect, and Diplostomum cultures which fail to show a high mitosis rate after 24 hours may be abandoned. This results in an immense saving of time and enables a rapid screening of media and culture conditions to be made.

Results with colchicine must however be interpreted with caution. A high mitotic count indicates a high rate of cell multiplication and serves as an excellent criterion for that particular phase of development - but that phase only; it does not necessarily imply that the medium or the cultural conditions which were found suitable for maintaining rapid nuceli multiplication are necessarily suitable for the later phases, some of which are synthetic phases involving materials other than those concerned in nuclear synthesis.

Results with Diplostomum cultured in vitro illustrate this point. In yolk media, the mitotic rate is moderately high and active spermatozoa ultimately develop. Further development beyond this stage i.e. egg-shell formation fails to occur. These later phases of development involve cytoplasmic synthesis of great quantities of protein pointing to the necessity of food requirements of a high order, whereas earlier phases such as spermatogenesis

require reorganization of the embryonic cells without intensive synthesis of new cytoplasmic material. Therefore the failure of the egg-shell formation phase to develop in Diplostomum indicates that although the culture medium selected could satisfy the requirements up to spermatogenesis, it could not meet the enormous demands of the egg-shell production stages. Similar results have been obtained with Diphyllobothrium fragments cultured in concentrated embryo extract (Smyth, 1958).

Therefore it may be concluded that a high mitotic count can be used to predict that the test medium will be suitable for development up to spermatogenesis. This conclusion is generally borne out by the results in Table III.

The question of time sequence is of particular importance in developmental studies, and in this respect each criterion is of double value. To satisfy a criterion, a certain developmental feature must not only appear, but it must do so within the same time range as it does in vivo. It is well known that with some helminths the maturation time required varies with the host so that precise definition of this may not always be possible. The same organism may behave differently, mature slower or produce fewer eggs in different hosts. Nevertheless, a range of in vivo patterns can be established and attempts made to achieve in vitro development within these limits; serious

Table III. Criteria recommended for the recognition of developmental phases in *Diplostomum phoxini*.

PHASE	TIME IN HOST (hrs)	CRITERION RECOMMENDED	METHOD OF DETECTION
1. Cell multiplication	0 - 24	mitoses counts	aceto-orcein squashes after colchicine treatment
2. Body shaping	24 - 48	division into regions	direct observations on living material and aceto-orcein squashes
3. Organogeny	12 - 24	appearance of testes & uterus primordia	squashes or whole mounts
4. Early gameto-	36 - 40	appearance of 'rosette' & 'comma' stages in spermatogenesis	squashes
5. Late gameto-	40 - 48	appearance of mature spermatozoa	squashes or unstained teases
6. Egg-shell formation & vitellogenesis	55 - 60	presence of egg-shell precursors in vitelline cells	histochemical tests on whole specimens: diazo $^{+}$ve, catechol $^{+}$ve
7. Oviposition	60 - 72	appearance of fully-formed egg	direct observations on living material or catechol-treated whole mounts

time discrepancies from the developmental pattern may be taken to indicate abnormal development. Such marked differences between *in vivo* and *in vitro* developmental times are characteristic of early developmental studies. Thus, Ferguson (1940) matured *Posthodiplostomum minimum* *in vitro* and found that maturation as judged by the appearance of eggs required 4 days compared with 35 - 40 hours *in vivo*. Weinstein & Jones (1956) in their recent interesting work on the development of *Nippostrongylus muris* *in vitro* similarly found that the fifth stage larva took 3 - 4 times longer to develop *in vitro* than *in vivo*.

In trematodes the most satisfactory criteria have proved to be mitosis, spermatogenesis, maturation of vitellaria and egg-shell formation in that order. In connection with the use of the latter criterion, it must be stressed that although the trematode used in the present study, and probably the majority of trematodes, form a quinone tanned egg, it has been found that the egg-shell of several forms (Smyth & Howie, 1957), while giving a reaction with the diazo reagent, gives a negative test for polyphenol oxidase with catechol. This suggests the existence of an alternative enzyme system in trematodes for the stabilization of egg-shell protein. Failure to give a positive catechol reaction must thus be interpreted cautiously and confirmatory tests carried out with the diazo reagent. In those trematodes examined, none has yet been

found whose egg-shell material does not give a positive diazo reaction.

Since the pattern of genitalia maturation in digenetic trematodes and pseudophyllidean cestodes is basically the same (Smyth, 1956b) closely similar criteria may be used for the maturation phases of these two groups. This is borne out by the fact that the above criteria, with slight modification have also been applied successfully to *in vitro* studies of *Diphyllobothrium dendriticum* (Smyth, 1958).

SUMMARY

1. Maturation of the metacercaria of Diplostomum phoxini follows a pattern which may be divided into the following phases: (1) cell multiplication; (2) body shaping; (3) organogeny; (4) early gametogeny; (5) late gametogeny; (6) egg-shell formation and vitello-:genesis; (7) oviposition.

2. Cytological or histochemical criteria have been developed by means of which each developmental phase may be recognized. Development in vitro may be considered comparable with that occurring in vivo if the same phases are completed within the same incubation time and without the appearance of cytological abnormalities.

3. The cell multiplication phase is characterized by high mitotic activity. This activity in vitro can be evaluated by incubating larvae after 24 hours' culture for 4 hours in 0.01 per cent colchicine in Tyrode's solution and comparing the number of mitoses seen in an aceto-orcein squash preparation with those found in a fluke matured in vivo for the same period. Use of this criterion enables the growth-producing powers of a medium to be assessed after 24 hours' culture.

4. Phases 2 - 4 are characterized by the appearance of the genitalia, the rudiments of which are readily recognized in aceto-orcein squashes or whole mount preparations.

5. The presence of spermatids and mature spermatozoa (phases 4 - 5) may be detected in aceto-orcein squashes or in fresh preparations. The quantity and activity of spermatozoa also serve as important criteria.

6. Recognition of phase 6, egg-shell formation, is carried out by means of diazo reagents which give red or orange-red reactions with the phenolic egg-shell precursors in the vitellaria, as seen in whole mount preparations. Abnormal egg-shell development is reflected in the failure of the vitellaria to give a normal diazo reaction.

7. The appearance of eggs which are macroscopically normal constitutes the final maturation phase. Early detection of eggs (especially abnormal ones) is facilitated by the use of the diazo reaction or the catechol test for polyphenol oxidase.

8. Embryonation and hatching of eggs to produce morphologically normal infective miracidia serve as final criteria.

9. Criteria for the maintenance of the adult condition are also considered.

AXENIC DEVELOPMENT OF *DIPLOSTOMUM PHOXINI* IN COMPLEX VISCOUS MEDIA

SECTION III

INTRODUCTION

The processes taking place in the maturation of Diplostomum phoxini from the metacercarial stage to the adult form involves the rapid synthesis of protein and nucleic acids before genitalia can develop and egg production begin. One of the main problems, therefore, in the cultivation of this trematode, is to develop a medium which is capable of supplying growth materials of this order. In a previous section (Section I) attempts were described to supply these growth requirements by the use of glucose-salines augmented by horse serum and chick embryo extract. With these media an increase in the mitotic rate was obtained but spermatozoa and ova failed to develop. It was suggested that in such liquid media only those substances may be used which are absorbed through the cuticle and therefore the flukes were obtaining insufficient nutriment to permit the maturation of the genitalia.

Since the maturation of Diplostomum is essentially a problem of tissue growth this suggests that the use of embryonic materials, which provide a rich source of growth substances, might be successful as culture media and because this trematode is an avian parasite, avian embryonic material is indicated.

The data given in this section are concerned with the development achieved from the cultivation of Diplostomum phoxini in embryonic media.

Before the growth promoting powers of a medium can be assessed, a 'standard' must be set up with which in vitro observations can be compared. In the case of Diplostomum, the 'standard' taken is the pattern of development of this trematode within a susceptible host, namely the duck; development has been divided into several phases (Section II, Table III) which are recognizable by the use of various cytological and histochemical criteria.

The aim of the present experiments was to obtain development in vitro which not only satisfied these criteria but did so within approximately the same time range.

MATERIAL AND METHODS

Metacercariae of D. phoxini were obtained from the brains of minnows. The metacercariae were removed from the fish by the aseptic technique previously described (Section I) and 10 - 20 transferred to suitable containers for culture.

Prior to the preparation of the media, the bench and the glass cover-plate, under which all operations were carried out, were swabbed with propylene glycol in order to minimize air-borne infection of the cultures (MacKie & McCartney, 1950).

Details of the methods of preparation of the various media, together with the type of culture vessel used and the conditions of culture, are given in the appropriate sections below.

Each culture, after opening, was examined for the presence of bacteria within the medium. This was carried out by making a thin smear of the medium on a slide, drying in air, staining for two minutes in 1 per cent aqueous methylene blue, rinsing in water and air drying. Using this procedure, bacteria, if present in the medium, could be detected readily by microscopic examination of the smear. All results obtained from infected cultures have been omitted since such observations are unreliable.

At intervals, during cultivation, flukes were removed

for histological and cytological examination. General observations on the flukes were made either by microscopic examination of live mounts or by direct aceto-orcein squashes. For the detection of mitoses, the flukes were incubated in Tyrode's solution plus 0.01 per cent colchicine for 4 hours at 40°C and aceto-orcein squashes prepared. Vitellogenesis and egg-shell formation was followed by fixing the specimens in 70 per cent ethanol and staining in diazo or catechol reagents (for details of the methods employed see Section II). Thus a complete picture of the progress of maturation of the genitalia in different media was available for comparison with the pattern of development which is found to occur in vivo.

MATURATION IN VITRO

I. Yolk Media

"The high biological value of egg proteins and especially of the yolk proteins, makes the egg an especially important source of nutriments for animals and man....." (Romanoff & Romanoff, 1949.) Since it is known that yolk proteins contain amino acids in sufficient quantity and variety to supply the developing embryo with the necessary material for tissue building, it seemed possible that yolk, used as a medium, might also supply sufficient nutriment to permit the maturation of Diplostomum.

(1) Fertile Duck Egg Yolk

In incubating fertile eggs, many enzymes are detectable in the yolk after 3 days' incubation and by 5 - 6 days all the enzymes common to the vertebrate gut have appeared. Yolk of this age is rich in embryonic growth materials, particularly amino acids (Needham, 1931). It was decided, therefore, to culture the metacerciae in fertile duck egg yolk which had been incubated for varying periods of time.

Preparation of incubated Fertile Duck Egg Yolk

Eggs: it was arranged that 10 fertile duck eggs of the required age of incubation were available.

Sterile glassware required:

15 crystallising dishes covered with large Petri dish lids.....wrapped.

15 20 ml. pipettes.....plugged in tubes.

15 10 ml. pipettes.....plugged in tubes.

15 1oz. universal containers.

15 6" x ¾" rimless culture tubes.....plugged.

Miscellaneous equipment required:

Large enamel dish.

Rubber tube with plugged mouthpiece.

Iodine in absolute ethanol (1 grm. in 100 ml.)

2" Petri dish for iodine.

1 paint brush.

1 scalpel.

Procedure:

1. The crystallising dishes were unwrapped and laid out in a row on the bench.

2. The surface of the pointed end of an egg was painted with alcoholic iodine and allowed to dry. A vertical lamp was used to hasten drying.

3. Using a scalpel, a cavity was chipped open on the surface of the egg, sufficient to permit the liquid yolk to flow out into a crystallising dish, the albumen being retained within the shell.

4. This process was repeated until each yolk had been

transferred to a separate crystallising dish.

5. Using a 20 ml. pipette, each yolk was transferred to a universal container.

6. The universal containers were centrifuged at 1944 R.C.F.* for 30 minutes.

7. The supernatant liquid (7 - 8 ml. per container) was transferred to the rimless culture tubes, using 10 ml. pipettes, and the tubes plugged with cotton wool.

Fertile duck egg yolk of 8, 10 and 12 days' incubation was tested. 8 - 10 tubes of each medium were prepared and the cultures incubated in a waterbath at 40°C.

Results: a summary of the results is given in Table I.
8-day incubated yolk - the mitotic rate rose steadily till the 3rd day after which it fell to zero. 'Rosettes' and 'commas' (see Section II, Table III) were visible on the 2nd day but no further development occurred although the flukes were alive on the 8th day when the last culture was examined. On the 5th day, abnormal cells, similar in appearance to the 'giant' polyploid cells observed by Smyth (1952) and Clegg (1957), were observed.
10-day incubated yolk - active spermatozoa appeared in the seminal vesicle after 87 hours' cultivation; no further

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*Relative Centrifugal Force.

Table I. Results of cultivation of D. phoxini in Incubated Fertile Duck Egg Yolk (D.E.Y.) at 40°C

MEDIUM	DEVELOPMENT OBSERVED AT VARIOUS INTERVALS							REMARKS
8day Incubated D.E.Y.	Hrs	19	44	67	90	120	144	168	120-168Hrs abnormal cells observed
	Count	15,19,9,7	74,56,66,66	68,61,95,74	10,24,25,15	16,10,34	3-5/fluke	0-2/fluke	
	Stage	-	rosettes; commas	no sperm	no sperm	no sperm	no sperm	no sperm	
	No.ex.	5	12	15	23	17	14	24	
10 day Incubated D.E.Y.	Hrs	19	40	60	87	112	144	168	at 87 hrs sperm active in seminal vesicle
	Count	-	47,4,34,49	18,52,72,63	67,60,66	15-25/fluke	10-20/fluke	-	
	Stage		many rosettes	early sperm	active sperm	active sperm	abnormal cells visible	abnormal cells visible	
	No.ex.		14	20	15	20	10	4	
12 day Incubated D.E.Y.	Hrs	19	43	66	90	120	144	168	at 120 hrs fatty degenera-:tion present
	Count	24,19,12,18	15,29,16,20	18,9,10,12	4,5,1,4	0,0,1,0	-	-	
	Stage	-	no sperm	no sperm	no sperm	no sperm			
	No.ex.	8	9	13	11	9			

Abbreviations: Count - mitosis count after 4 hours' treatment in colchicine; the four highest counts obtained are given.

Stage - development observed in fresh preparations and/or aceto-orcein squashes.

No.ex. - total number of flukes examined at each time interval.

development occurred and by the 6th day, cytological examination revealed the presence of 'giant' polyploid cells.

12-day incubated yolk - the mitotic rate remained low during the 5-day culture period. Spermatozoa were absent and fatty degeneration of the flukes occurred on the 5th day.

Of these three media, the 10-day incubated yolk gave significantly better results (Fig. 1). Though few in number, the spermatozoa obtained in this medium were morphologically normal and showed activity. However, the initial mitotic rate was low and the appearance of spermatozoa at 87 hours was unsatisfactory when compared with the 40 - 48 hours required *in vivo*.

(2) Infertile Hen Egg Yolk

From the foregoing experiments it was apparent that although active spermatozoa were obtainable, the pattern of development was decidedly abnormal. Since, in incubating eggs the yolk proteins are quickly being utilized by the developing embryo, it was decided to examine the effect of culturing the flukes in infertile, unincubated egg yolk. Hen egg yolk was used as duck eggs were not readily obtainable. Previous delay caused by incubation of the eggs was avoided and thus a more rapid screening of media was made possible.

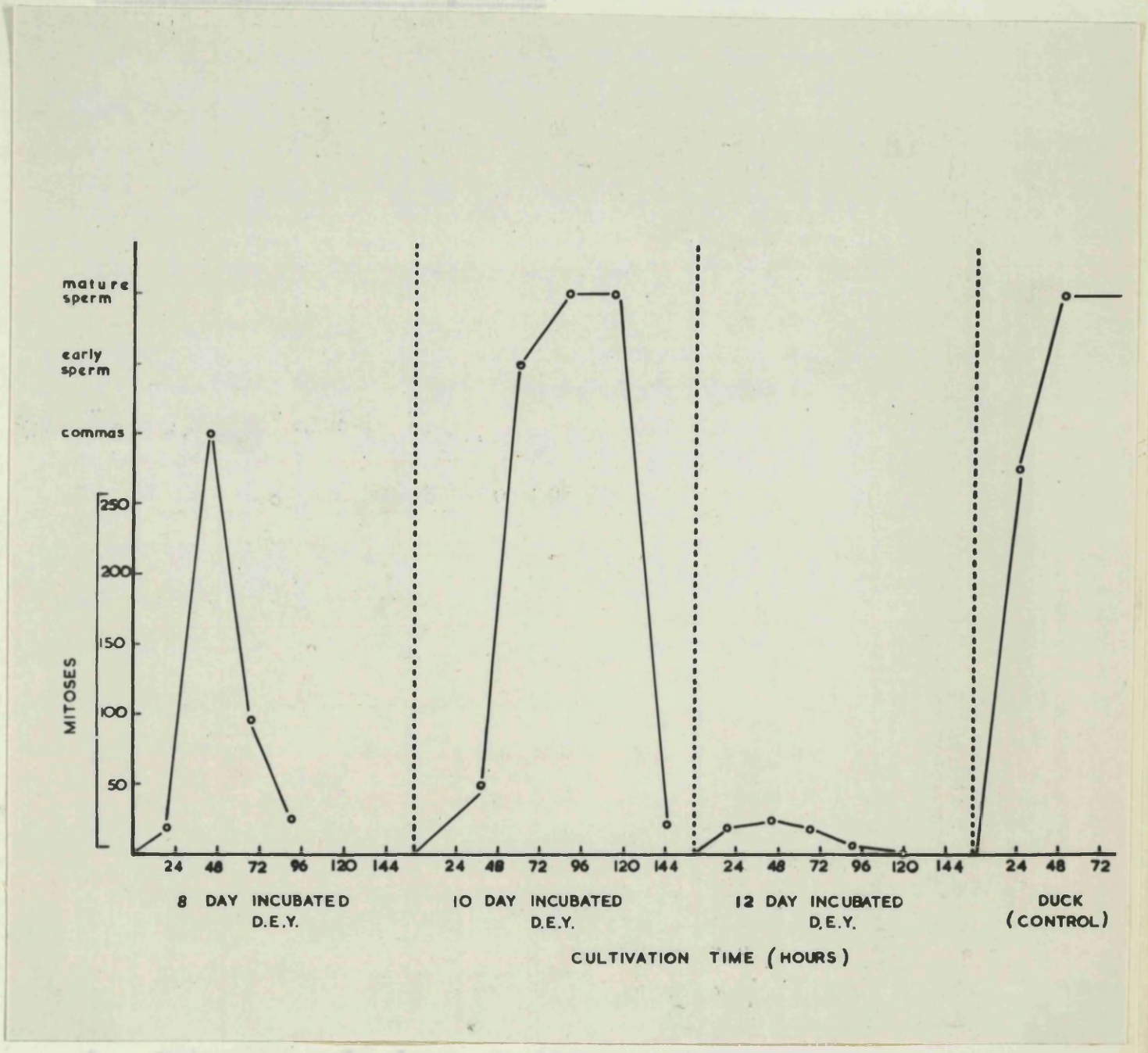

Fig. 1. Comparative development of *D.phoxini* in Fertile Duck Egg Yolk (D.E.Y.) after different periods of incubation.

Preparation of unincubated infertile Hen Egg Yolk

Eggs: 5 fresh infertile eggs were procured.

Sterile glassware required:

6 crystallising dishes covered with large Petri dish lids.....wrapped.

6 20 ml. pipettes.....plugged in tubes.

12 5 ml. pipettes.....plugged in tubes.

Miscellaneous equipment required:

Large enamel dish.

Rubber tube with plugged mouthpiece.

Iodine in absolute ethanol (1 grm. in 100 ml.)

2" Petri dish for iodine.

1 paint brush.

1 scalpel.

Procedure:

1. 100 ml. of sterile Tyrode* was prepared.

2. The crystallising dishes were unwrapped.

3. The entire surface of the egg was painted with alcoholic iodine and allowed to dry.

4. Using a scalpel, a cavity was chipped open on the surface of the egg sufficient to permit the entire contents of the egg to flow out into a crystallising dish.

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*Tyrode - modified (as in Section I) to contain 1% glucose.

It was desirable to keep the yolk membrane intact.

5. This process was repeated with each egg.

6. Using a 20 ml. pipette, the albumen was removed and discarded.

7. Using a 5 ml. pipette, 5 ml. of Tyrode was added to each dish containing yolk and the dish was rotated to ensure thorough mixing of the yolk and Tyrode.

8. 5 ml. of the yolk-Tyrode medium was transferred to each culture vessel.

Using this yolk-Tyrode, hereafter referred to as yolk medium, various culture conditions were examined in an attempt to find the optimum. They were:

(a) Various culture vessels.

(b) Revolving of cultures.

(c) Introduction of physical resistance within the medium.

(a) Culture vessels

Three different culture vessels were tested. They were:

1. 2½" x ½" rimless culture tubes plugged with cotton wool.

2. 1oz. universal containers.

3. 5 ml. eccentric-nozzle hypodermic syringes.

Ten cultures of each type of culture vessel were prepared and incubated at 40°C.

Results (Table II): in these three experiments the mitotic rate greatly exceeded that previously obtained in other media. In each case, mature spermatozoa and ciliated uterus developed although their time of appearance varied from 88 hours in the case of the universal containers and syringes to 120 hours in the rimless culture tubes (Fig. 2).

It was therefore concluded that of these three culture vessels, the universal containers were the most suitable and their ease of handling minimized the risk of bacterial contamination during the preparation of the media.

(b) Revolving of cultures

Although the initial mitotic rate obtained in these yolk cultures approached that found in vivo (Fig. 2), the time required by the flukes to complete spermatogenesis in vitro (i.e. 88 hours) was unsatisfactory and the number of spermatozoa which ultimately developed was poor. As it was possible that this might be due to the accumulation of metabolic waste materials within the immediate vicinity of the flukes, the effect of revolving the cultures during incubation was examined.

The metacercariae were transferred to 5 ml. amounts of yolk medium in universal containers and 8 cultures (and a stationary control) set up and revolved at 4 revolutions per minute on a modified roller-tube apparatus and incubated in the oven at 40°C.

Table II. Results of cultivation of D. phoxini in Unincubated Hen Egg Yolk (H.E.Y.) at 40°C

MEDIUM & CULTURE CONDITIONS	DEVELOPMENT OBSERVED AT VARIOUS INTERVALS					REMARKS
5 ml. H.E.Y. open tube	Hrs	18	42	66	90	120	at 120 hrs - uterus visible
	Count	27,87,95,90	76,33,45,49	11,3,32,16	27,8,29,5	1,1,0,1	
	Stage	-	no sperm	commas	early sperm	active sperm	
	No.ex.	9	9	15	12	10	
5 ml. H.E.Y. loz containers	Hrs	18	41	65	88	120	at 88 hrs - ovary and ciliated uterus visible
	Count	80,71,53,123	260,210,100,85	80,35,56,47	-	-	
	Stage	-	commas	early sperm	active sperm		
	No.ex.	15	20	27	40		
5 ml. H.E.Y. syringes	Hrs	19	41	65	88	120	at 88 hrs & 120 hrs ciliated uterus visible
	Count	90,82,83,36	141,49,147,83	26,22,24,25	-	-	
	Stage	-	rosettes	no sperm	mature sperm	mature sperm	
	No.ex.	7	6	12	6	10	

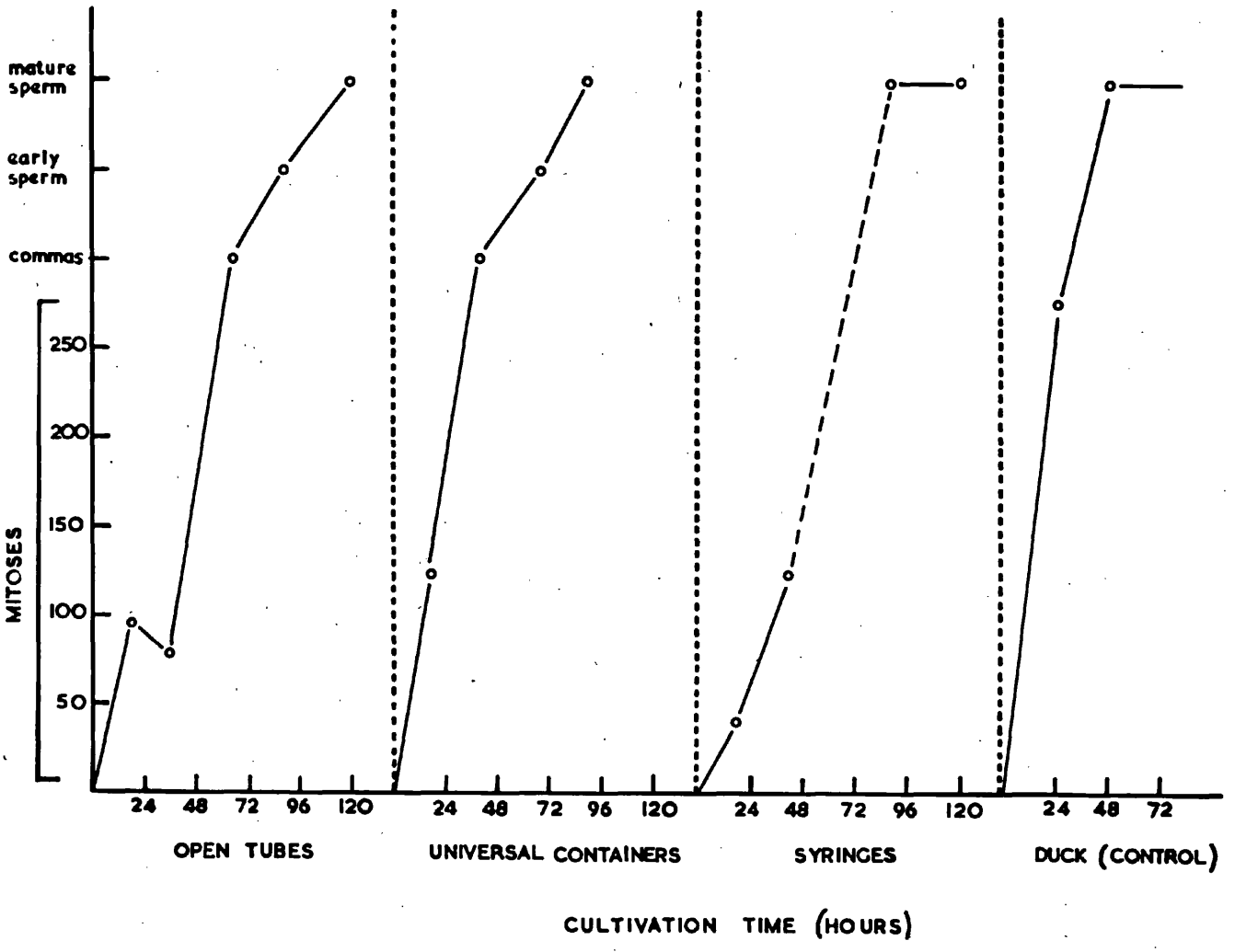

Fig. 2. Comparative development of D.phoxini in Infertile Hen Egg Yolk using various culture vessels.

No development beyond the 'rosette' stage occurred (Table III) and the flukes were all dead at 90 hours. In the control experiment, active spermatozoa were visible at 90 hours.

When suitable conditions prevail, it has been observed that the flukes will readily adhere to the culture vessel. Continuous revolving of the cultures apparently prevented the flukes from doing this.

(c) Physical resistance within medium

In an attempt to provide a suitable surface to which the flukes might adhere while revolving, cotton wool was placed in the bottom of the containers, a few drops of horse serum were added and the containers autoclaved. The serum coagulated thereby anchoring the cotton wool within the medium. 5 ml. of yolk medium was added as before and 6 cultures (and a control) revolved at 4 revolutions per minute at 40°C.

In the revolving cultures the larvae were dead within 71 hours (Table III); in the control, active spermatozoa were visible at 90 hours.

Concurrently a similar experiment was set up but in this case the cultures (10) were incubated in a stationary condition in a waterbath at 40°C.

The result (Table III) was a substantial improvement

Table III. Results of cultivation of D. phoxini in unincubated Hen Egg Yolk (H.E.Y.) at 40°C

MEDIUM & CULTURE CONDITIONS	DEVELOPMENT OBSERVED AT VARIOUS INTERVALS					REMARKS
	Hrs	18	41	67	90	144 - 168	control experiment: sperm active at 90 hrs
H.E.Y. revolving	Count	49,64,63,51	51,31,28,64	2,19,5,9	all dead	-	
	Stage	-	rosettes	only 5 alive; no sperm			
	No.ex.	10	10	5	-		
H.E.Y. serum & cotton wool revolving	Hrs	18	40	70	90	144 - 168	control experiment: sperm active at 90 hrs
	Count	150,134,145,100	41,29,31,46	all dead	-	-	
	Stage	-	-	-			
	No.ex.	14	18	-			
H.E.Y. serum & cotton wool stationary	Hrs	18	42	65	90	144 - 168	at 90 hrs: active sperm in seminal vesicle; ciliated uterus
	Count	130,180,110,130	107,92,71,150	-	-	-	
	Stage	-	no sperm	10% with mature sperm	10% with active sperm	no sperm uterus well developed	
	No.ex.	17	27	28	36	33	

in the time of appearance of mature spermatozoa, i.e. 65 hours (Fig. 3) compared with the 90 hours previously required. It was believed that, in this experiment, the cotton wool offered some physical resistance to the worms wriggling in the culture thereby permitting them to find a certain optimum level in the medium. This suggestion was borne out by the fact that during recovery of the flukes from the medium, many were found adhering firmly to the strands of cotton wool.

In order to exclude the possibility of this beneficial effect being due to the presence of the coagulated horse serum in the medium, cultures were prepared in which the serum was added as before, this time omitting the cotton wool base and the cultures set up at 40°C. 1) revolving and 2) stationary. In the former case, spermatozoa failed to develop and the worms died within 4 days; in the latter case, active spermatozoa were observed at 90 hours.

II. Albumen Medium

"Albumen is a very watery solution of protein, containing only the most negligible traces of fats and lipids, but a great many water soluble substances such as carbohydrate in various forms, protein breakdown products...." (Needham, 1931).

To find the effect of using infertile hen egg albumen as a culture medium, metacercariae were introduced into

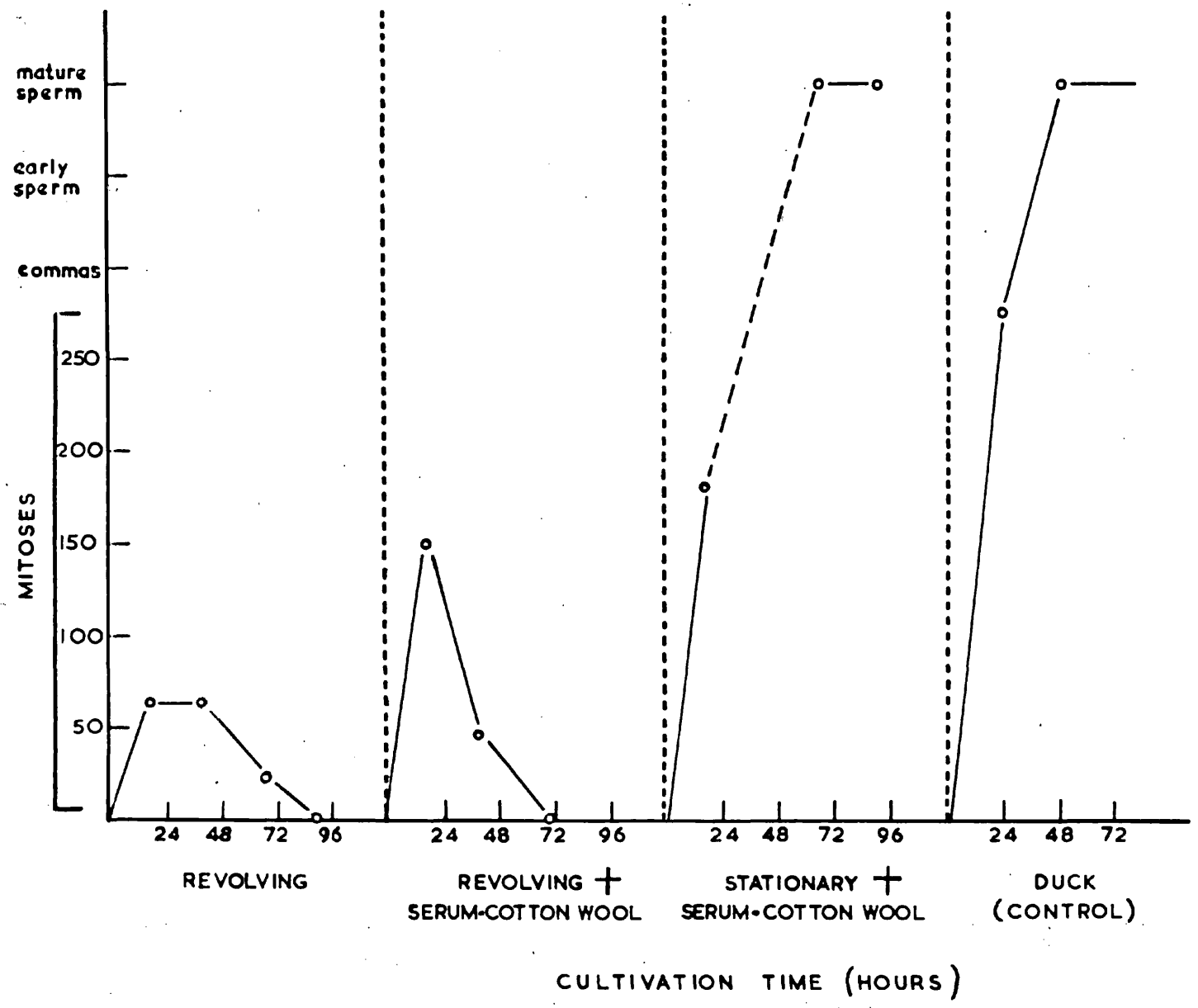

Fig. 3. Comparative development of D.phoxini in Infertile Hen Egg Yolk under varying conditions of culture.

albumen to which Tyrode had been added (1 part Tyrode: 3 parts albumen) and cultured in universal containers and large Carrel flasks in the oven at 40°C.

The number of mitoses observed at 18 hours exceeded 200 in some cases, falling to 3 by 66 hours' incubation. Although some flukes were alive on the 4th day, cytological examination revealed the absence of mitoses or spermatozoa.

III. Yolk Medium Plus Albumen

Since albumen appeared to possess the property of stimulating mitosis, it was decided to culture Diplostomum in yolk medium to which albumen had been added.

Preparation of yolk-albumen medium

The method employed was similar to that used in the preparation of yolk medium. In this case, approximately 3 ml. of albumen was added to each universal container, the yolk medium prepared as before and transferred to the containers.

In the first of these yolk-albumen experiments, 10 ml. of yolk medium was added to the albumen and the cultures incubated in a waterbath at 40°C.

Results (Table IV): the degree of maturation achieved in this medium surpassed that previously obtained.

Male genitalia - the number of mitoses observed at 17 hours (> 200) approached that found in organisms matured in vivo

and in one fluke mature spermatozoa were observed in the testes after 40 hours' cultivation. Within 64 hours, 85 per cent of the flukes were producing active spermatozoa; no difference was observed either in the activity or in the number of these spermatozoa when compared with those produced *in vivo*.

Female genitalia - after 64 hours' cultivation, the ciliated uterus, ovary and Mehlis' gland were clearly visible in live preparations. At 88 hours, a few flukes were producing eggs, of which some were found free in the medium; in only one of the flukes was a vitelline reservoir observed, although in the others, a few vitelline cells were visible. Diazo tests for the presence of egg-shell precursors (Section II) all gave negative results. By the 5th day, most flukes possessed a vitelline reservoir and eggs were present in the uterus. The vitelline cells had increased in number and although these gave a reaction with the diazo test, the colour produced was abnormal. In these specimens the colour developed in the vitellaria was yellow-brown compared with the red or orange-red reaction which is produced in *in vivo* matured specimens. The vitelline cells in the uterus region ranged from brown to black in appearance in fresh preparations. This was thought to be due to the premature 'tanning' of the vitelline cells which had failed to group themselves round an ovum and indicated an abnormality of egg-shell formation.

The eggs produced in this medium were abnormal; the shell was not uniform (Fig. 4) and showed marked thickenings in several places. Egg production continued up to the 6th day when the last culture was examined.

A second series of experiments using yolk-albumen medium was carried out. In (a) the amount of yolk medium added was increased to 20 ml. to give semi-anaerobic conditions; in (b) the amount of albumen used was decreased to 1 ml. to which was added 10 ml. of yolk medium. The cultures were incubated at 40°C in a waterbath. The results obtained in both cases (Table IV) were similar to those of the previous experiment.

In order to simulate the intestinal movement which occurs in the definitive host, fresh cultures (3 ml. albumen and 10 ml. yolk medium) were clamped into a shaking machine in a waterbath at 40°C and shaken continuously (about 100 times per minute) throughout the incubation period. The result was a distinct improvement in the time of appearance of the vitelline reservoir and in the number of flukes producing eggs (Table IV) compared with that observed in similar cultures incubated in a stationary condition.

Table IV. Results of cultivation of D. phoxini in Yolk-Albumen medium at 40°C

MEDIUM & CULTURE CONDITIONS	DEVELOPMENT OBSERVED AT VARIOUS INTERVALS							REMARKS
		Male Genitalia			Female Genitalia			
	Hrs	17	40 - 42	64 - 66	88 - 90	64 - 66	88 - 90	112 - 114	
13 ml. Yolk-Albumen Stationary	Stage	mitoses - 210,220,150,130	mature sperm in one; commas	85% with active sperm in seminal vesicle	active sperm	ciliated uterus; ovary; Mehlis' gland; diazo -ve	vitelline reser-:voir in one; eggs in medium; 3 have 2-3 eggs; diazo -ve	eggs and vitel-:line reservoir in many; tanned vitellaria; diazo +ve (brown)	6th day: active sperm and eggs
	No.ex.	10	29	23	24	23	24	22	
23 ml. Yolk-Albumen Stationary	Stage	-	commas	active sperm in seminal vesicle	active sperm	ciliated uterus	a few with 2-6 eggs each; tanned vitel-:laria; diazo +ve (Brown)	-	90 hrs: those flukes with eggs were free in medium
	No.ex.		22	12	18	12	18		
10 ml. Yolk- 1 ml. ½ Albumen Stationary	Stage	-	rosettes	active sperm in seminal vesicle	active sperm	ciliated uterus; ovary; diazo -ve	vitelline cells moving in uterus; egg in one; diazo +ve (brown)	1-6 lightly tanned eggs/ fluke; diazo +ve (brown)	7th day: active sperm and eggs present
	No.ex.		20	15	25	15	25	20	
13 ml. Yolk-Albumen Shaken	Stage	mitoses - 300,190,170,160	one with early sperm; rosettes	active sperm in seminal vesicle	active sperm	uterus; vitel-:line cells	vitelline reser-:voir in all; many with 1-2 eggs; diazo +ve (yellow-brown)	eggs; vitel-:laria; diazo +ve (yellow-brown)	
	No.ex.	11	17	26	20	26	20	18	

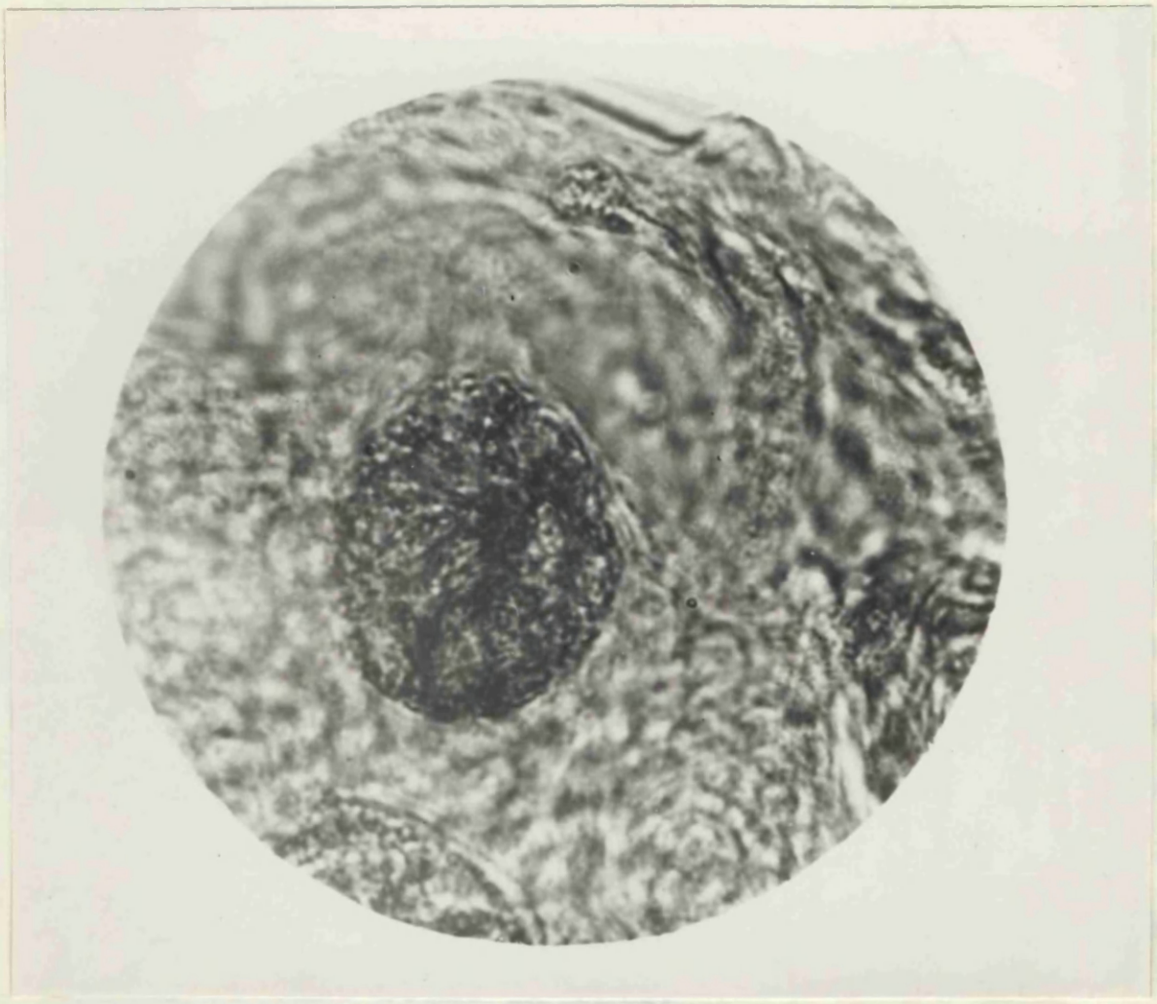

Fig. 4. Egg in fluke matured in yolk-albumen medium. Note the abnormal shell formation. (oil immersion).

VITELLOGENESIS AND EGG-SHELL FORMATION IN VITRO

From the foregoing experiments it was apparent that, although maturation of the male and the female genitalia with the eventual production of eggs occurred in yolk-albumen medium, the vitellaria and eggs were abnormal. In these specimens, the colour developed with the diazo reagent in the vitellaria was yellow to brown in sharp contrast to the brilliant red reaction which is obtained with flukes matured *in vivo*. This indicated an abnormal synthesis of the protein or phenolic precursors of the shell material.

In an attempt to supply the missing substance or substances, various extracts or compounds were added to the yolk-albumen which now served as the basic medium for all further experiments. The main criterion adopted was the colour developed with the diazo reagent and the aim of the following experiments was to obtain a colour reaction similar to that found *in vivo*.

I. Yolk-Albumen Plus Tissue Extracts

The effect of adding tissue extracts to yolk-albumen was examined. The extracts used were: 1) Raw liver extract; 2) Duck embryo extract; 3) *Schistocephalus* extract.

1. Raw liver extract

This was added at 1%, 2% and 5% levels to the yolk-albumen and the cultures incubated in a stationary

condition at 40°C in a waterbath. In this experiment, and throughout the remainder of the in vitro experiments, all chemical additions to the basic medium were made up in Tyrode (without glucose) in the stated amounts, autoclaved and sterile glucose added to give a final concentration of 1 per cent. This solution was then added, as before, to the yolk and the yolk-albumen prepared.

Results (Table V):

1% - active spermatozoa were present in great numbers at 88 hours. The vitelline reservoir was visible in only one fluke and the diazo reaction was yellow-brown. Eggs were not produced.

2% - in only one fluke did mature spermatozoa develop; these were inactive and few in number.

5% - fatty degeneration of the worms occurred at 39 hours; no growth or development had taken place.

Although spermatozoa production was excellent when raw liver extract was added at a 1 per cent level, egg production was inhibited.

2. Duck embryo extract

Eleven day-old duck embryo extract was prepared as described in Section I and 5 ml. added to each yolk prior to the preparation of the basic yolk-albumen medium. The cultures were incubated at 40°C on the shaker.

Table V. Results of cultivation of D. phoxini in Yolk-Albumen plus Raw Liver Extract (R.L.E.) at 40°C

MEDIUM & CULTURE CONDITIONS	DEVELOPMENT OBSERVED AT VARIOUS INTERVALS						REMARKS
		Male Genitalia				Female Genitalia	
	Hrs	16	39	63	88	63	88
Yolk-Albumen + 1% R.L.E. stationary	Stage	mitoses-210,220,180,190	early sperm in one; others - rosettes	85% with mature sperm in testes	active sperm in seminal vesicle	ciliated uterus; diazo -ve	vitelline reservoir in one; no eggs; diazo +ve (yellow -brown)
	No.ex.	15	25	25	16	25	16
Yolk-Albumen + 2% R.L.E. stationary	Stage	mitoses-150,110,160,120	a few mito-:ses; no foliation of anterior end	one with mature sperm in testes	no sperm	-	-
	No.ex.	11	16	16	24		
Yolk-Albumen + 5% R.L.E. stationary	Stage	mitoses-19,34,17,28 sluggish	no growth; no mitoses	-	-	-	-
	No.ex.	14	15				

fatty de-:generation occurring at 39 hrs

Results (Table VI): egg production continued from the 5th day to the 9th day when the last culture was examined. Although the number of eggs produced exceeded that obtained in flukes matured in vivo, the egg-shell formation was abnormal. The ovum, with its large nucleus and nucleolus was clearly visible within each egg. The diazo test proved negative in every case. This was believed to be correlated with the excessive egg production by the flukes whose vitelline reserves were thereby rapidly depleted.

3. Schistocephalus extract

Since the plerocercoids of Schistocephalus solidus contain the necessary precursors of the vitelline cells of that organism (Smyth, 1954b) it was decided to add an extract of these in an attempt to provide the substances essential for the production of normal vitellaria in Diplostomum. The extract was prepared as follows:

The plerocercoids were removed aseptically (Smyth, 1946) and 6 transferred to a Petri dish containing 20 ml. Tyrode; the larvae were finely chopped with scissors and left overnight at room temperature. 5 ml. of this mixture (i.e. Tyrode and Schistocephalus fragments) were added to each yolk and the yolk-albumen plus extract cultures set up in a shaker at 40°C.

Results (Table VI): the eggs produced were abnormal and with the diazo test the vitellaria gave a yellow-brown

reaction.

II. Yolk-Albumen Plus Reducing Agents

The addition of these tissue extracts failed to supply the missing factor which would permit normal vitellaria production and egg-shell formation. It was thought possible that the oxidising conditions existing in the cultures were not conducive to normal egg production. Therefore, it was decided to examine the effect of adding reducing agents to the basic medium for the purpose of producing a reducing environment within the medium. Two such agents were l-cysteine hydrochloride and ascorbic acid. The former was tested at a 25 mgm. per cent level and the latter at 8 mgm. per cent. The cultures were shaken intermittently (7 seconds on, 120 seconds off) at 40°C.

Results (Table VII): l-cysteine hydrochloride - at 89 hours, only one fluke had an egg present; the egg-shell was abnormal. The diazo test was negative. Ascorbic acid - egg production was poor. The diazo test was negative although, in live preparations of the flukes, the vitelline cells were clearly visible.

III. Yolk-Albumen Plus Casein Hydrolysate

By this stage it was thought that the failure of the vitellaria to give a normal colour reaction with the diazo test was due possibly to the fact that either some essential amino acid was missing from these media or was present only

Table VII. Results of cultivation of D. phoxini in Yolk-Albumen plus Reducing Substances at 40°C

MEDIUM & CULTURE CONDITIONS	DEVELOPMENT OBSERVED AT VARIOUS INTERVALS							REMARKS
		Male Genitalia				Female Genitalia		
	Hrs	21	41	-	89	89	144	
Yolk-Albumen + 25 mgm.% Cysteine HCl shaken	Stage	>200 mitoses	-	-	active sperm in seminal vesicle	egg in one; vitelline cells; diazo -ve	-	at 89 hrs - active sperm surrounding egg
	No.ex.	6			12	12		
Yolk-Albumen + 8 mgm.% Ascorbic Acid shaken	Stage	-	commas; rosettes	-	active sperm in seminal vesicle	eggs in two flukes diazo -ve	no eggs; vitelline reservoir and vitellaria in one; diazo -ve	
	No.ex.		15		18	18	16	

in negligible amounts. Since casein provides an excellent source of amino acids (Hawk, 1954) it was decided to augment the amino acid content of the basic medium by the addition of casein hydrolysates.

Casein hydrolysate, obtained from the acid hydrolysis of casein (containing 50% amino acids, 50% lactose) was added to the basic medium at 2% and 4% levels and the cultures incubated on a shaker at 40°C.

Results (Table VIII):

2% - abnormal eggs were present (9 eggs in one fluke) at 120 hours and vitellaria production exceeded that so far obtained in any culture (Fig. 5); reaction with the diazo test was reddish-brown.

4% - the mitosis rate was low and by 40 hours the flukes were dying.

Casein hydrolysate, obtained from the enzyme hydrolysis of casein and with a low carbohydrate content, was added at a concentration of 1.5 per cent. (To compensate for possible changes in osmotic pressure, half-strength Tyrode was used to make up the basic medium.) The cultures were shaken at 40°C.

Results (Table VIII): the eggs produced were not 'tanned' but were yellowish in appearance. One egg was distinctly membranous in texture but unfortunately the shell collapsed

Table VIII. Results of cultivation of D. phoxini in Yolk-Albumen plus Hydrolysates at 40°C

MEDIUM & CULTURE CONDITIONS	DEVELOPMENT OBSERVED AT VARIOUS INTERVALS							REMARKS
		Male Genitalia				Female Genitalia		
	Hrs	17	40	89	120	89	120	
Yolk-Albumen + 2% Casein Hydrolysate (acid hydro-:lysis) shaken	Stage	mitoses - 220,200,340, 210 early sperm in one	rosettes	-	active sperm	-	eggs - 1 fluke with 9 eggs; tanned vitel-:laria; diazo +ve (brownish)	vitellaria-greatest amount so far produced
	No.ex.	7	12		15		15	
Yolk-Albumen + 4% Casein Hydrolysate (acid hydro-:lysis) shaken	Stage	mitoses - 110,77,65,38	sluggish discarded	-	-	-	-	
	No.ex.	5	4					
Yolk-* Albumen 1.5% Casein Hydrolysate (enzyme hydrolysis) shaken	Stage	-	early sperm; commas	active sperm in seminal vesicle	active sperm	vitelline reservoir; no eggs; catechol -ve	eggs (untanned) diazo +ve vitel-:laria (slightly reddish-brown)	120 hrs - one egg had shell almost 'normal' in appearance
	No.ex.		4	12	15	12	15	

*½ strength Tyrode used to compensate for possible change in osmotic pressure.

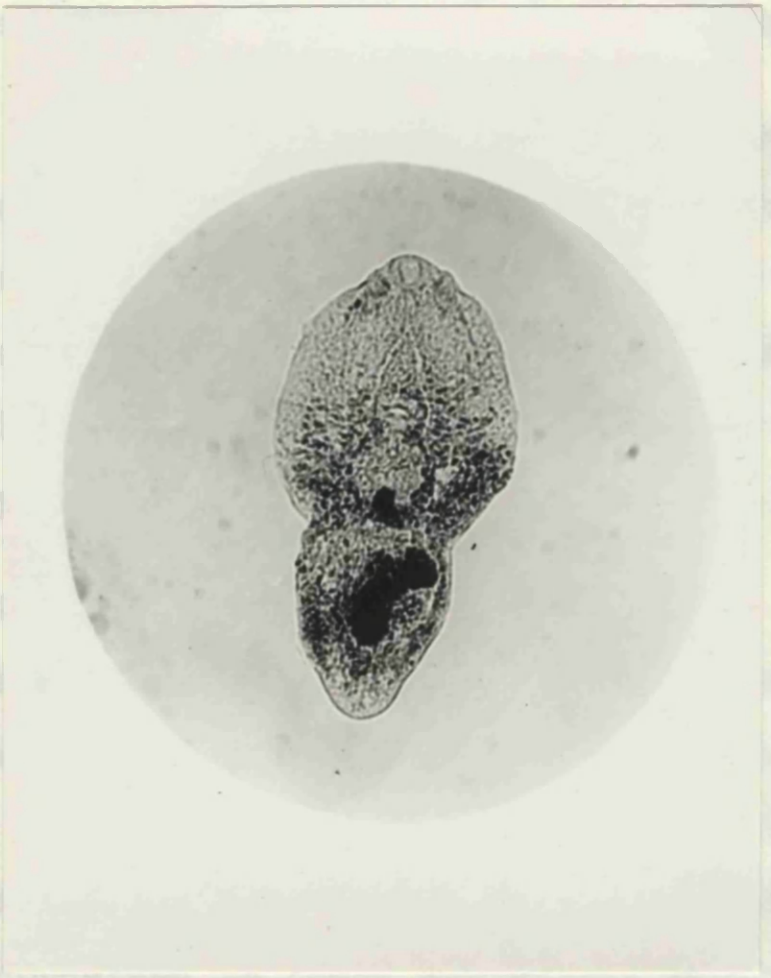

Fig. 5. Fluke matured in yolk-albumen plus casein hydrolysate. (X 100).

on fixation and therefore no photographic record is available. In another egg, although the shell was abnormal, the vitelline globules showed a tendency to flow together, which condition occurs in normal egg-shell formation. With the diazo test the vitellaria gave a yellow-brown reaction.

The addition of 1.5 per cent casein hydrolysate to yolk-albumen medium resulted in a slight improvement in the formation of the egg-shell. Although the vitellaria in fresh specimens appeared normal, their chemical composition, as judged by their reaction with the diazo reagent, was decidedly abnormal.

IV. Yolk-Albumen Plus Gelatine

As has been stated previously (Section II), the vitelline cells have many properties similar to those cells of other quinone tanning systems such as that secreting the byssus of Mytilus (Smyth, 1954b; Brown, 1952) and since the protein concerned in these systems is known from X-ray analysis to be collagen, it is possible that the precursors of the egg-shell material in Diplostomum may be closely related to those required in other collagen forming systems.

Gelatine, a breakdown product of collagen-containing tissues, was added to the basic medium in five different concentrations:

(a) 2%, 4% and 10% - cultured at 40°C on the intermittent shaker.

(b) 3% and 5% - cultured at 40°C in the stationary condition.

The results (Tables IX and X) were as follows:

(a) 2% - one of the flukes examined at 89 hours gave a 'normal' colour reaction with the diazo test (Fig. 6); the amount of vitellaria present, however, was poor. The following day, another fluke which had copious vitellaria present, gave an orange-red reaction (Fig. 7); eggs present in the uterus were abnormally 'tanned'. By the 7th day, live mount examination revealed a general grey appearance of the posterior lobe due to the presence of a large mass of vitelline cells; with these cells the diazo reaction was reddish-brown.

4% - abnormal eggs were produced and the diazo reaction was yellow-brown.

10% - in two of the flukes, a faint red reaction was obtained; the eggs were abnormal.

(b) 3% - eggs were absent; in two flukes the diazo reaction was faint red.

5% - at 89 hours 20% of the flukes examined developed a faint red colour with the diazo test and by the 6th day of culture, 50% were giving an orange-red reaction.

Table IX. Results of cultivation of D. phoxini in Yolk-Albumen plus Gelatine at 40°C

MEDIUM & CULTURE CONDITIONS	DEVELOPMENT OBSERVED AT VARIOUS INTERVALS					REMARKS
	Hrs	17	89	120	168	
Yolk-Albumen + 2% gelatine shaken	Stage	early sperm in 2	active sperm; vitelline reservoir and vitel-:laria; 1-3 eggs/fluke + eggs free in medium; catechol -ve; diazo +ve (normal colour in one)	1-5 eggs/fluke; vitellaria abundant; diazo +ve - in one (with 5 eggs) the colour reaction was orange-red	general grey appearance in posterior end due to mass of vitelline cells; some tanned vitellaria; diazo +ve - reddish brown colour in 2	N.B. normal diazo reac-:tion at 89 hrs
	No.ex.	10	55	45	24	
Yolk-Albumen + 4% gelatine shaken	Stage	-	active sperm; 2 flukes with 1 egg each; vitel-:laria abundant; diazo +ve (yellow-brown)	-	-	
	No.ex.		20			
Yolk-Albumen + 10% gelatine shaken	Stage	> 200 mitoses	active sperm; vitelline reservoir; vitellaria abundant; 2 flukes each with 2 eggs; diazo - 2 +ve (faint red), rest -ve	-	-	N.B. abun-:dant vitellaria, yet only 2 diazo +ve
	No.ex.	7	20			

Table X. Results of cultivation of D. phoxini in Yolk-Albumen plus Gelatine at 40°C

MEDIUM & CULTURE CONDITIONS	Hrs	DEVELOPMENT OBSERVED AT VARIOUS INTERVALS			
		65	89	120	144
Yolk-Albumen + 3% Gelatine stationary	Stage	diazo -ve	active sperm; no eggs; malachite green -ve (7 exam.) catechol -ve (12 exam.) diazo - 14 examined - only 1 +ve (brown)	-	no eggs; diazo - only 1 +ve (faint red)
	No.ex.	22	33		20
Yolk-Albumen + 5% Gelatine stationary	Stage	-	active sperm; no eggs; no vitelline reservoir; a few vitelline cells a round adhesive organ diazo - +ve in 3 (faint red)	-	egg in one; diazo-50% +ve (orange-red)
	No.ex.		16		20
Yolk-Albumen + 1% Gelatine + 0.75% Casein Hydrolysate shaken	Stage	active sperm in seminal vesicle	-	active sperm; no eggs; no vitelline reservoir, vitellaria present; diazo -ve	eggs and vitellaria; diazo +ve (brown)
	No.ex.	19		20	23

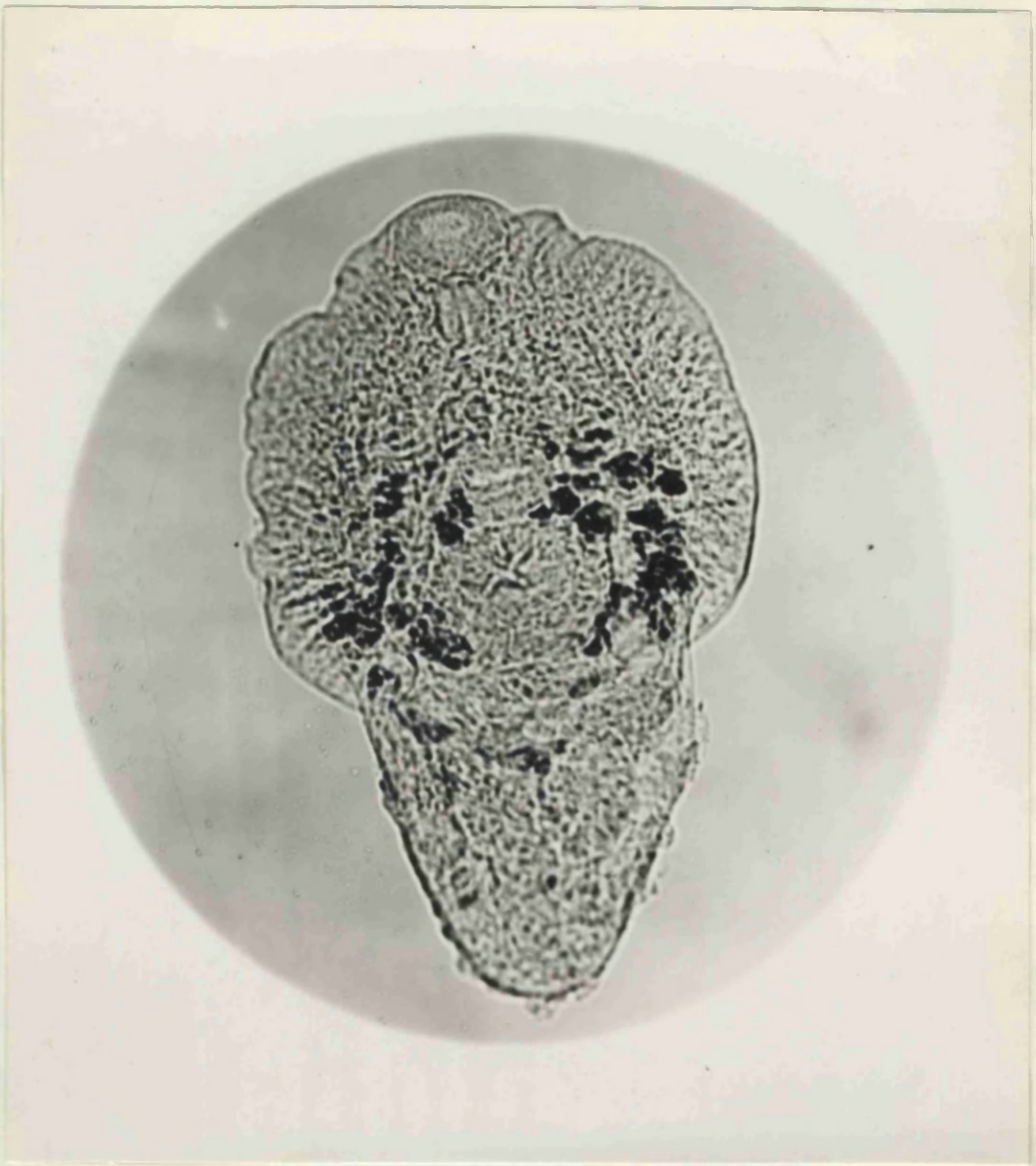

Fig. 6. 'Normal' diazo reaction given by fluke matured in yolk-albumen plus gelatine. Note - sparse vitellaria. (X 100).

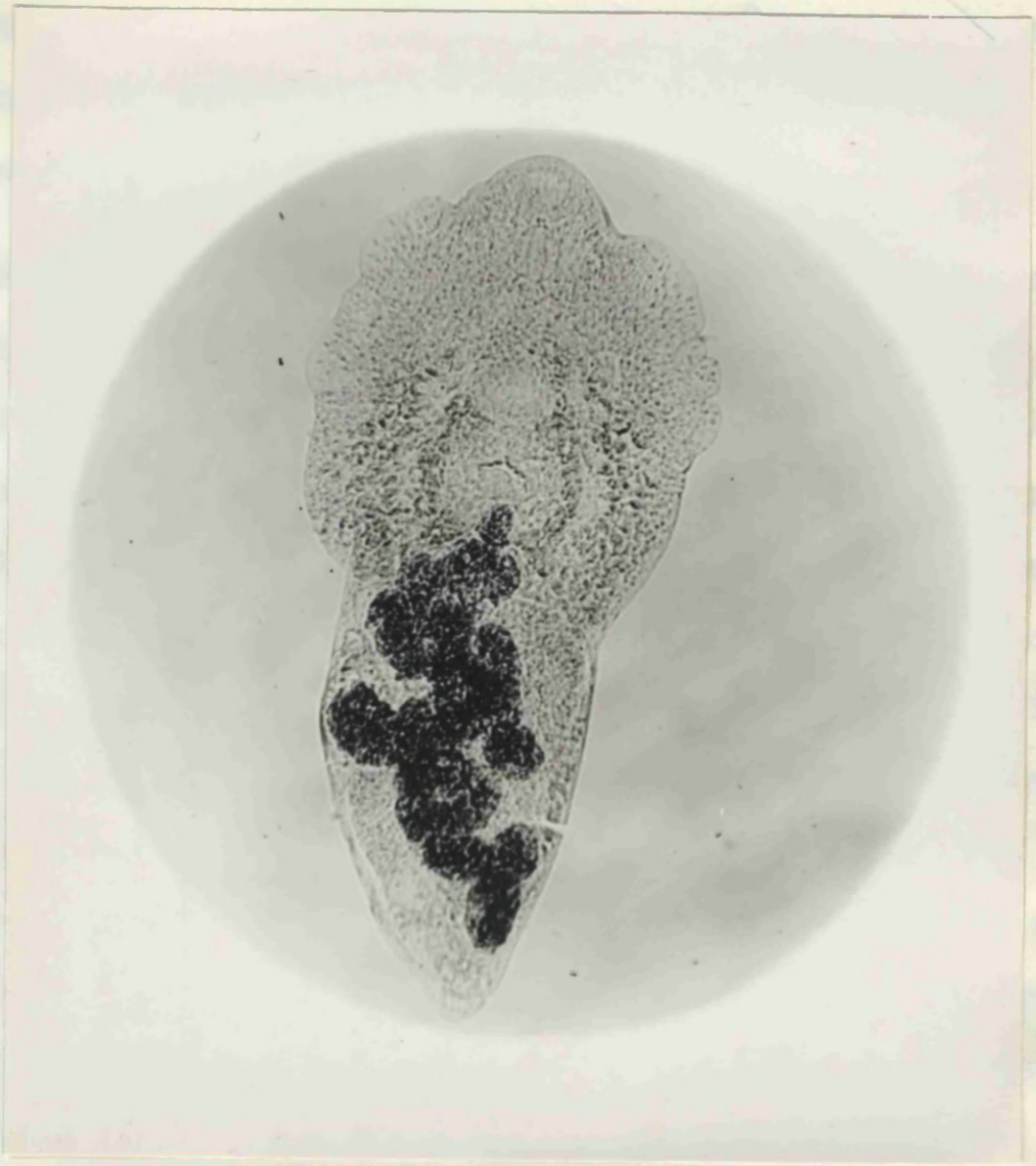

Fig. 7. Abnormal egg-shell formation in fluke matured in yolk-albumen plus gelatine. The eggs and vitellaria gave an orange-red reaction with the diazo test. (X 100).

In a later experiment, 1% gelatine and 0.75% casein hydrolysate (from enzyme hydrolysis) was added to the basic medium and the cultures incubated at 40°C on the intermittent shaker. The diazo reaction at 144 hours was brown; the eggs were abnormal (Table X).

From these experiments, it was apparent that the addition of gelatine to yolk-albumen medium resulted in a striking improvement in the normality of the chemical composition of the vitellaria as judged by the colour reaction obtained with the diazo test, although the eggs produced exhibited abnormal shell formation. With one exception (4% gelatine), at each concentration of gelatine examined, one or more flukes were found whose vitellaria gave a reaction ranging from faint red to orange-red and in one case (2% gelatine) a 'normal' colour was obtained. This was suggestive that the precursors of the egg-shell material in Diplostomum did consist of a collagen type of protein.

V. Yolk-Albumen Plus Proline and/or Hydroxyproline

In gelatine, the amino acids proline and hydroxyproline, together with glycine, predominate (Hawk, 1954). As hydroxyproline is absent (Hawk, 1954) and proline is present in only a small amount in yolk-albumen (Romanoff & Romanoff, 1949), it appeared possible that these might be the amino acids which are essential for normal vitellaria formation in Diplostomum.

L-proline and l-hydroxyproline were added, separately and combined, to the basic medium at different concentrations. The following experiments were set up and the cultures incubated at 40°C on the intermittent shaker:

Experiments - 1. 1% l-proline; 2. 0.9% l-hydroxyproline;
3. 0.5% l-proline plus 0.5% l-hydroxyproline;
4. 0.25% l-proline plus 0.25% l-hydroxyproline.

The results were as follows (Table XI) using the colour reaction of the vitellaria with the diazo test as the criterion:

Experiment 1 - approximately 15 per cent of the flukes examined gave a faint red colour. Two flukes gave a reaction approaching the 'normal' on the 5th day of culture. Eggs were absent.

Experiment 2 - in only one fluke was a faint red colour obtained throughout the 5-day culture period; the eggs were abnormal.

Experiment 3 - after 89 hours' cultivation, approximately 12 per cent of the flukes gave an orange-red reaction. After 120 hours, an orange-red to dark red reaction was observed in 50 per cent of the flukes examined. Abnormal eggs were produced (Fig. 8).

Experiment 4 - in one fluke the colour developed approached the 'normal' (Fig. 9); of the others, 50 per cent gave

Table XI. Results of cultivation of D. phoxini in Yolk-Albumen plus Proline and/or Hydroxyproline at 40°C

MEDIUM & CULTURE CONDITIONS	DEVELOPMENT OBSERVED AT VARIOUS INTERVALS		
	Hrs	88	120
Yolk-Albumen + 1% Proline shaken	Stage	active sperm; one with eggs; diazo - 2 +ve (faint red)	no eggs; vitellaria; 11 catechol - all -ve; 42 diazo - 6 gave faint red colour 2 gave almost normal reaction
	No.ex.	17	60
Yolk-Albumen + 0.9% Hydroxy-:proline shaken	Stage	active sperm; vitelline reservoir; 1-2 eggs in a few; diazo +ve - in 1 the colour was faint red	2-4 eggs/fluke; vitellaria; diazo +ve (brown)
	No.ex.	25	
Yolk-Albumen + 0.5% Proline + 0.5% Hydroxy-:proline shaken	Stage	no eggs; no vitelline reservoir; vitellaria scant; diazo - 31 examined, 4 gave orange-red reaction	4-9 eggs/fluke; vitellaria; vitelline reservoir; diazo - 50% +ve (orange-red to dark red)
	No.ex.	50	40
Yolk-Albumen + 0.25% Proline + 0.25% Hydroxy-:proline shaken	Stage	no eggs; vitellaria scant; diazo -ve	1-2 eggs in a few; vitellaria; vitelline reservoir; diazo - 50% +ve (orange-red) - 1 fluke gave almost normal colour
	No.ex.	27	30

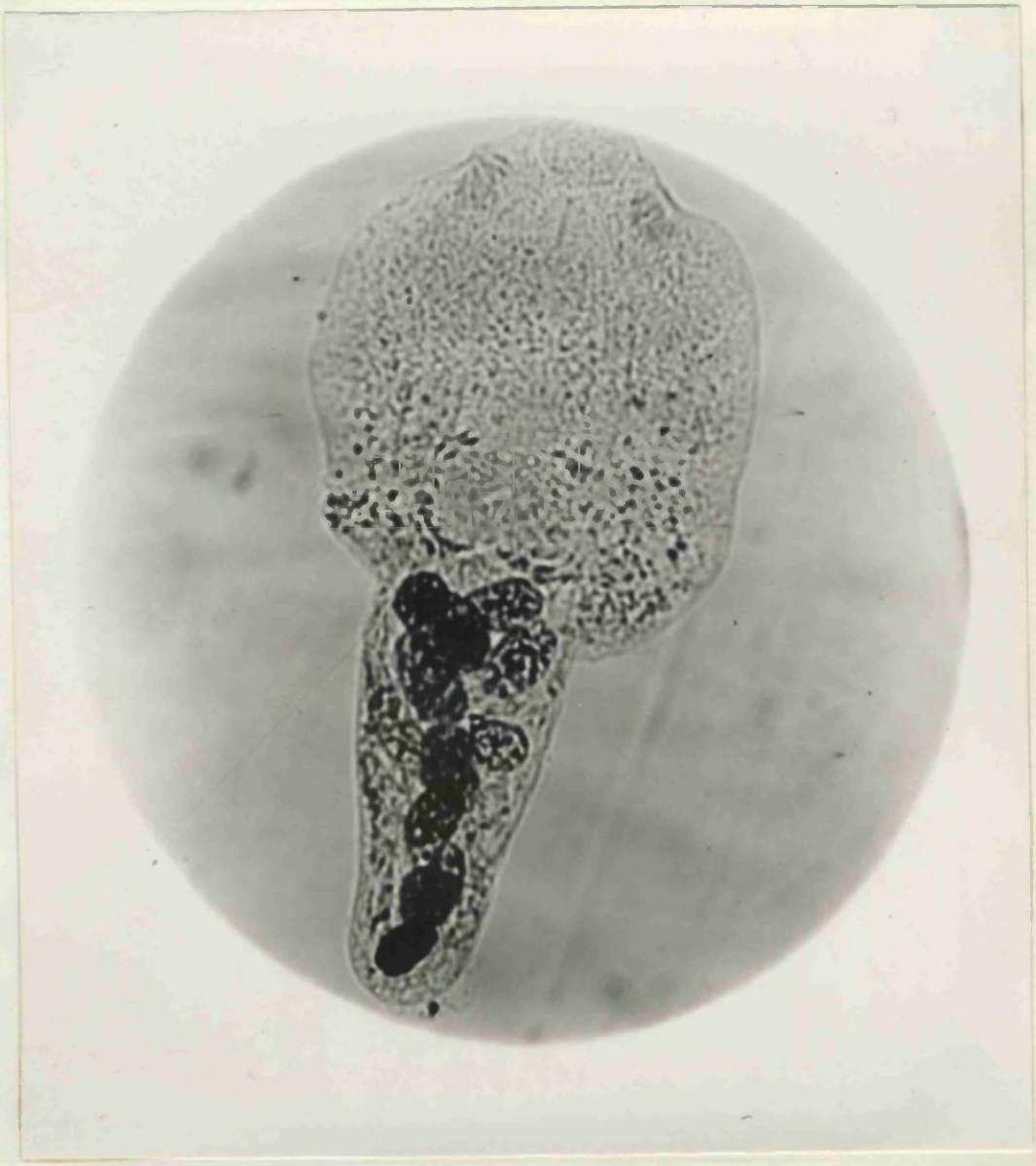

Fig. 8. Abnormal egg-shell formation in fluke matured in yolk-albumen plus proline-hydroxyproline. Note the almost complete lack of diazo +ve material in the vitellaria. (X 100).

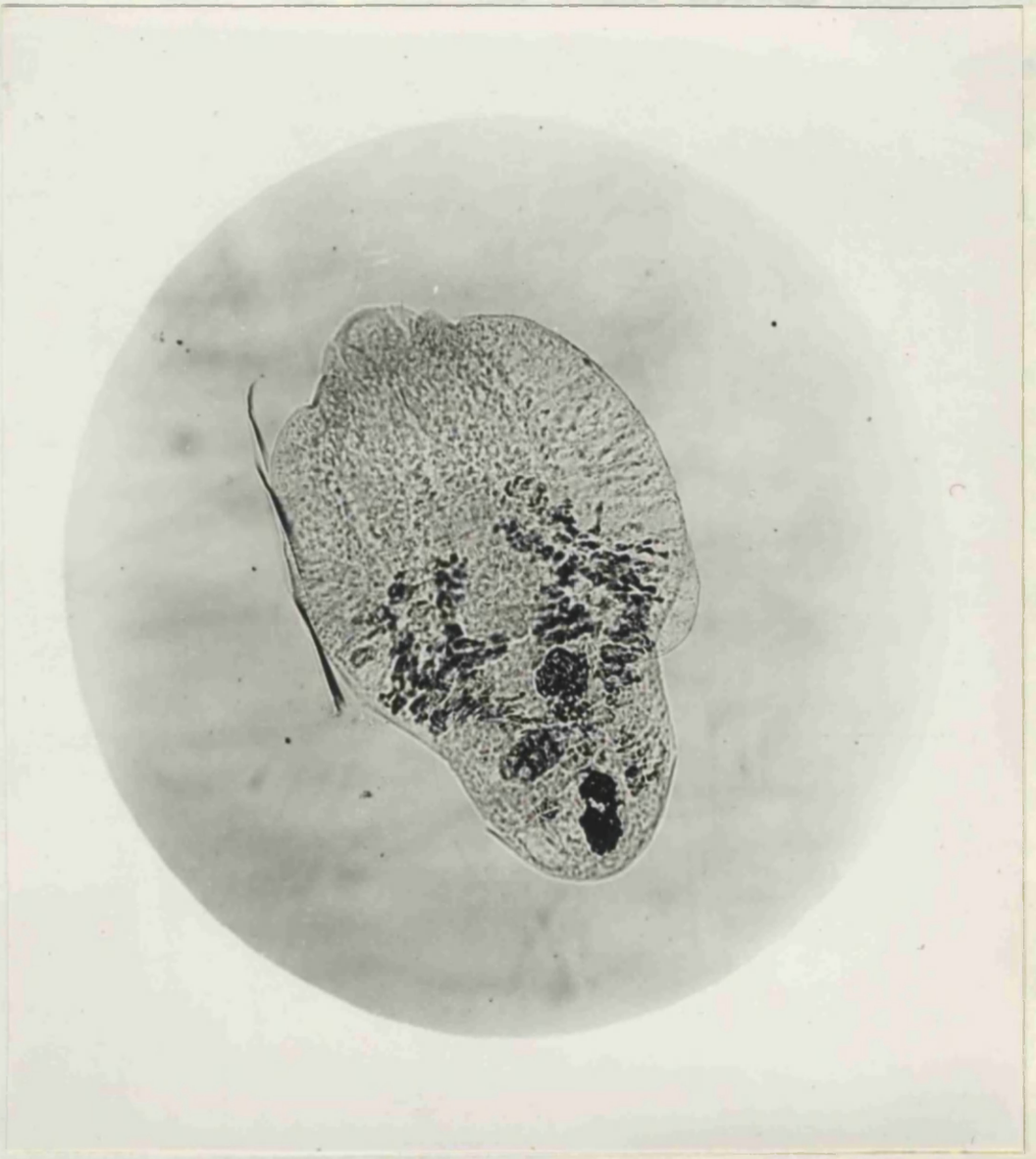

Fig. 9. Almost 'normal' diazo reaction with vitellaria of fluke matured in yolk-albumen plus proline-hydroxyproline. (X 100).

an orange-red reaction. Egg production was poor and those produced were abnormal.

Results obtained with the basic medium augmented by proline and hydroxyproline combined closely paralleled those from the gelatine experiments. This tended to indicate that these particular amino acids have an important function in the formation of the vitellaria in Diplostomum.

DISCUSSION AND CONCLUSIONS

Before the growth promoting powers of a medium can be assessed, a standard must be set up with which *in vitro* observations can be compared. In Section I, the pattern of development of *Diplostomum phoxini* in a susceptible host, namely the duck, was established. This pattern provided the required 'standard'.

In preliminary *in vitro* investigations, development to complete sexual maturity with the eventual production of spermatozoa and eggs is improbable. Therefore the criterion adopted was the 'normal' rate of cell division obtained *in vivo* and attempts were made to obtain development *in vitro* which satisfied this criterion.

Several media were investigated.

In salines, the metacercariae died within 18 hours' incubation owing to lack of an exogenous energy substrate.

In glucose media, the flukes survived well (4 - 6 days), but cytological examination after 72 hours showed that mitoses were occurring only in those flukes which were cultured in media to which serum had been added.

From these observations it was postulated that serum contained the basic nitrogenous products necessary to the fluke if it was to maintain the rapid rate of protein and nucleic acid synthesis which had been stimulated. The

absence of such a nitrogenous substrate resulted in a cessation of nuclear activity as soon as the fluke's endogenous reserves were exhausted, as was demonstrated in flukes cultured in glucose-salines (Fig. 10). However, the progressive fall in the rate of cell division in glucose-serum media together with the failure of spermatozoa and ova to develop suggested that, even in serum, the flukes were obtaining insufficient nutriment. It was believed that, in such liquid media, only those substances were utilized which could be absorbed through the cuticle. This hypothesis indicated that the use of solid nutriment might be more successful.

From the foregoing results, it was apparent that the provision of a more highly nutrient medium was essential, before the nutritional requirements of the flukes' various maturation processes could be satisfied.

In Section I, the 'standard' adopted was the 'normal' rate of cell division observed <u>in vivo</u>. For the later stages of maturation, culminating in the production of gametes and the materials forming the egg-shell and for the maintenance of the 'normal' adult condition, other criteria of a cytological or histochemical nature were essential. In Section II, the establishment and application of such criteria are described.

The aim of the <u>in vitro</u> experiments described in

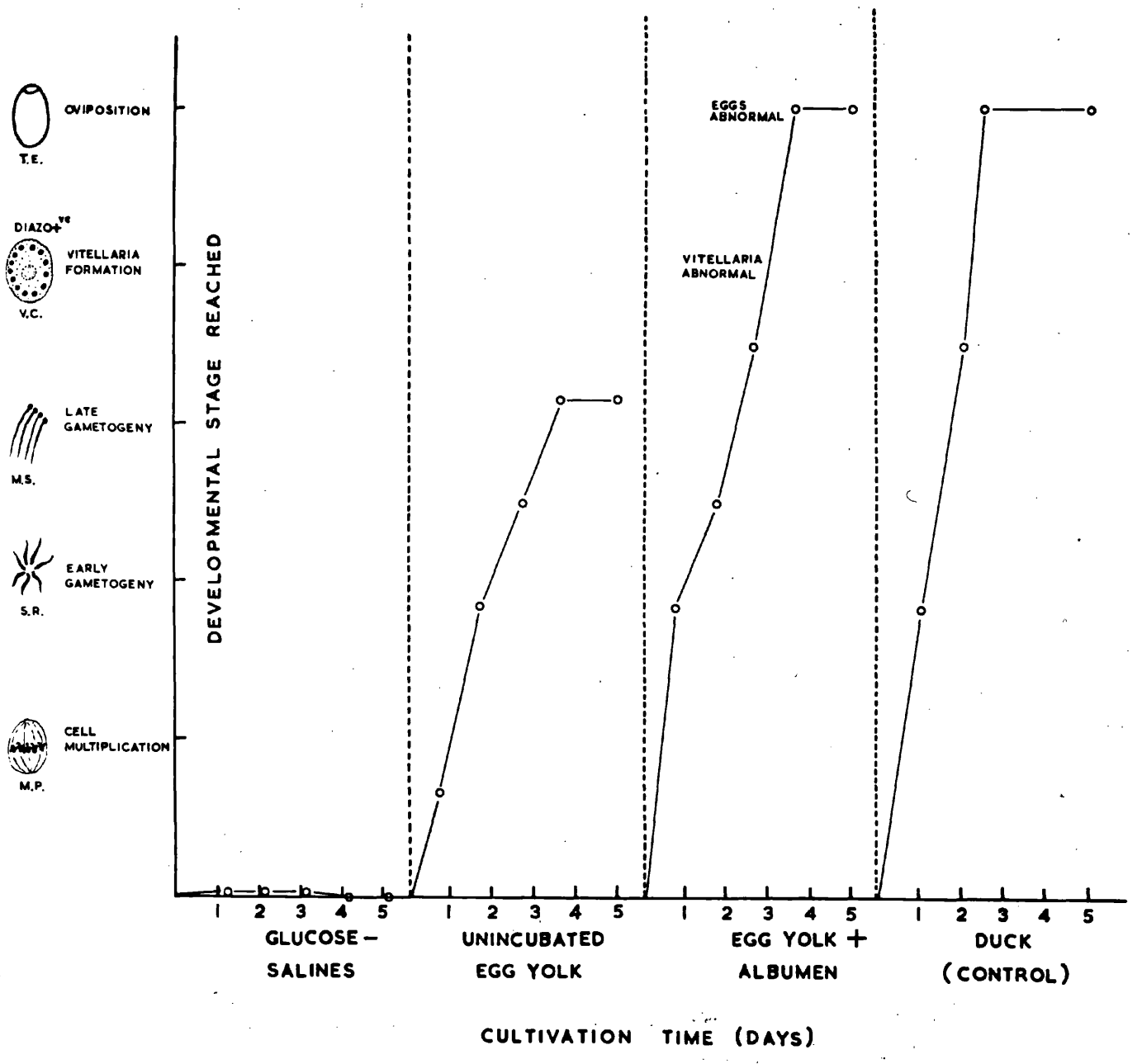

Fig. 10. Comparative development of D.phoxini in vitro and in vivo.

Section III, was to obtain development which satisfied these criteria within approximately the same time range.

I. Spermatogenesis and Oogenesis In Vitro

The effect of culturing Diplostomum phoxini in various viscous media are discussed below:

1. Incubated fertile Duck Egg Yolk

Eight, ten and twelve day incubated yolk was tested and of these three media, the 10-day incubated yolk gave the best results. In this medium, morphologically normal, active spermatozoa were produced at 89 hours, however, they were few in number. In the 8 and the 12-day incubated yolk, spermatogenesis did not occur.

It is of interest to note that spermatozoa were formed in a medium in which solid nutriment was available for the flukes. Examination of fresh specimens revealed the gut endings to be filled with yolk particles, thereby confirming that Diplostomum can ingest solid nutriment.

A satisfactory explanation cannot be found to account for the failure of spermatozoa to develop in 8 and 12-day incubated yolk. It may be of significance that the maximum intensity of protein catabolism and of protease activity in incubated fertile eggs occurs between the 8th and 9th day (Needham, 1931).

Another interesting feature is that although no glucose

was added to the yolk, the flukes survived from 5 - 7 days. Free glucose, however, is recorded as being present in yolk at approximately 0.7 per cent level and carbohydrate in the combined form (as mannose and galactose) at 0.3 per cent level (Romanoff & Romanoff, 1949). (Needham (1931) states the reverse, i.e. approximately 0.3 per cent is free glucose and 0.7 per cent is in the combined form.) By the 20th day of incubation, the percentage of free and combined carbohydrate falls to approximately 0.7 per cent (Needham, 1931). It would appear, therefore, that the yolk possessed a sufficiently high concentration of glucose to satisfy the flukes' requirements.

2. Infertile Hen Egg Yolk plus Tyrode

In this yolk medium, the mitotic rate exceeded that previously obtained in other media, moreover the uterus and mature spermatozoa were visible at 88 hours' cultivation. Unincubated yolk contains the full complement of embryonic growth materials and in this respect may be superior to incubated fertile yolk in which some of these growth materials have been utilized by the developing chick.

Attempts to shorten the period required by the flukes for the completion of spermatogenesis by revolving the cultures, were unsuccessful. A possible explanation is that the flukes, which, it has been observed, will adhere firmly to a suitable surface if available, were expending energy in attempting to attach themselves to the culture

vessel.

The introduction of cotton wool into the yolk medium resulted in the appearance of spermatozoa at 65 hours compared with the 90 hours previously required. During recovery from the medium, the flukes were found firmly attached to the strands of cotton wool, from which it was concluded that the cotton wool offered some physical resis-:tance to the flukes wriggling in the culture thereby permitting them to find a certain optimum level in the medium.

Yolk medium can evidently satisfy the nutritional requirements of the early maturation stages up to and including spermatogenesis, but fails to satisfy the enormous demands of the final maturation stages, i.e. vitellogenesis and egg-shell formation.

3. Albumen medium

In flukes cultured in albumen plus Tyrode, the number of mitoses observed after 18 hours' incubation exceeded 200, falling to 3 at 66 hours. Cytological examination showed the absence of spermatozoa. Albumen seemed to provide an excellent surface to which the flukes readily adhered and in many cases they were observed to penetrate the albumen.

It was apparent from the rate of cell division that the albumen possessed the property of stimulating mitosis. The

failure of this initial outburst of nuclear activity to culminate in the production of spermatozoa is probably due to the accumulation of metabolic wastes around the flukes with the consequent inhibition of their development.

4. Yolk medium plus albumen

The degree of maturation achieved in this medium surpassed that previously obtained (Fig. 10). A few flukes were producing eggs within 88 hours' cultivation and by the 5th day, most flukes exhibited a vitelline reservoir and vitellaria; eggs were also present in the uterus. However, diazo tests for the presence of egg-shell precursors gave an abnormal colour reaction and the egg-shell formation was also abnormal.

The beneficial effects observed when albumen is added to yolk medium could be due either to the chemical composition of the albumen or to its possessing suitable physical properties.

Albumen, on addition to Tyrode, was noted to have the property of stimulating mitosis, from which observation it appeared that the albumen was supplying some basic nitrogenous substances necessary for the fluke's rapid protein and nucleic acid synthesis. Similarly, the addition of albumen to yolk medium appeared to provide additional nutriment in that the number of spermatozoa produced was increased until it was similar to that

obtained in *in vivo* matured specimens.

With regard to the physical properties of albumen, the possible beneficial effect of the pH, which may range from 9.0 to 9.7, is not known and further investigation is required before any conclusions can be drawn.

General observations of the culture medium during recovery of the flukes, showed that optimum results were obtained when only the watery extract had been used and only when this had thoroughly intermixed with the yolk medium. If, in error, the more viscous portion of the albumen was used, the flukes attached themselves to this and their development was inhibited.

The introduction of shaking the cultures during incubation, for the purpose of simulating the intestinal movement which occurs in the duck, resulted in a distinct improvement in the time of appearance of the vitelline reservoir and in the numbers of flukes producing eggs when compared with similar cultures incubated in a stationary condition. The probable interpretation of these results is that shaking not only dispersed the accumulating metabolic wastes but also provided fresh 'surfaces' of nutriment for the flukes. Since, at this stage, it has not yet been found practical to change the medium during the incubation period, shaking the cultures has provided an invaluable solution to this problem. Intermittent shaking

has two advantages over constant shaking in that it (a) permits a short resting period during which the flukes may feed and (b) prevents overheating of the shaking mechanism which may be in constant use for several weeks or more.

II. Vitellogenesis and Egg-Shell Formation In Vitro

Attempts to achieve normal vitellaria production and egg-shell formation by the addition of various tissue extracts to yolk-albumen were unsuccessful. It is of interest to note, however, that egg production in flukes cultured in yolk-albumen plus duck embryo extract exceeded that observed in vivo.

Results obtained after attempts to procure a reducing environment within the basic yolk-albumen medium were poor. Nevertheless, further investigation of the effects of adding reducing agents to the cultures is indicated before any conclusions can be drawn.

A slight improvement in the formation of the egg-shell was observed when the basic medium was augmented by casein hydrolysate. Moreover, the vitellaria were abundant and the diazo reaction was reddish-brown (Table XII). The casein hydrolysate appeared to supplement the nitrogenous content of the basic medium, as was exhibited by the increase in the quantity of vitellaria, but failed to provide the essential substance required for normal vitellogenesis. However, the faint red tinge obtained

Table XII. Evaluation of culture media using the diazo test as a criterion.

MEDIUM	COLOUR DEVELOPED IN THE VITELLARIA AFTER TREATMENT WITH DIAZO REAGENT					
	YELLOW	BROWN	FAINT RED	ORANGE-RED	DARK RED	'NORMAL' BRIGHT RED
Duck (control)	-	-	-	+	+	+
Yolk-albumen	+	+	-	-	-	-
Yolk-albumen PLUS						
1. Casein hydrolysate	+	+	+	-	-	-
2. Gelatine	+	+	+	+	-	+
3. Proline	+	-	+	-	-	-
4. Hydroxyproline	+	+	+	-	-	-
5. Proline + hydroxy-:proline	-	-	+	+	+	-

with the diazo test suggested that the solution to the problem lay in the provision of an essential amino acid (or amino acids) which had so far been lacking, or which was present in only negligible amounts in all the media so far examined.

When gelatine was added to yolk-albumen, a normal diazo reaction was observed in one fluke and many others gave a reaction approaching normal (Table XII). This indicated an improvement in the normality of the chemical composition of the vitellaria and tended to confirm that the precursors of the shell material of Diplostomum consisted of a collagen type of protein.

Pursuing this possibility further, the amino acids - proline and hydroxyproline, which with glycine are predominant in gelatine (Hawk, 1954), were added to the basic medium. The results obtained paralleled those observed in the gelatine experiments (Table XII). This suggests that these particular amino acids have an important function in the formation of the vitellaria of Diplostomum and may indeed be the essential factors which all media, previously tested, have lacked.

The experiments described above, are as yet, at a very preliminary stage. Maturation of the male and the female genitalia with the subsequent production of eggs at 88 hours has been achieved in vitro. Since egg production

in vivo begins at 60 - 65 hours, it will be noted that development *in vitro*, at this stage in the experiments, lags by approximately 28 hours. Moreover, the vitellaria, although approaching the normal regarding quantity and chemical composition, require further improvement and the problem of normal egg-shell formation remains to be solved.

In the cultivation of *Diplostomum phoxini*, the outstanding problem that remains is the development of a medium which will provide suitable physico-chemical conditions and at the same time satisfy the requirements of the flukes' final maturation processes.

From the foregoing experiments, it would appear theoretically possible to obtain normal vitellaria and eggs in yolk-albumen augmented by gelatine or by proline-hydroxyproline, providing a suitable reducing environment could be achieved within the medium. Available evidence suggests that the vertebrate gut has a low oxygen tension, but the degree of oxygen tolerance *in vitro* which is permitted before abnormal development occurs is unknown. Owing to the existence of oxidising conditions in the media in which *Diplostomum* has been cultured, the polyphenol oxidase-protein complexes in the vitelline cells became prematurely oxidised with the result that the vitelline cells became 'tanned' and streamed from the genital pore; consequently the egg-shell was unable to form normally. Similar observations were made by Hopkins (1952) while

culturing Schistocephalus in a high oxygen tension environment. However, when cultured in semi-anaerobic conditions, Schistocephalus produced a high proportion of normal eggs. The importance of a reducing environment cannot be over-emphasised in the light of these observations with Diplostomum and Schistocephalus.

As a basic culture medium, yolk-albumen has several disadvantages. It is opaque, thereby making observation of the flukes during incubation impossible and consequently the cultures must be opened and the contents emptied into a Petri dish before an estimation of their development can be carried out. The variability in the chemical composition and physical properties (e.g. pH, Eh) of this medium, depending on its age and the condition of the hen at time of laying, renders stabilization of culture conditions impossible.

The use of such media of biological origin can only be expected to make limited contributions to the problem of helminth nutrition and the introduction of methods using chemically defined media to investigate these problems is clearly desirable.

SUMMARY

1. Metacercariae of Diplostomum phoxini were removed aseptically from the brains of minnows and cultured at 40°C in various complex viscous media under sterile conditions. Development of the cultured worms, as revealed by cytological and histological examination, was compared with that of worms matured normally in the duck.

2. In hen egg yolk medium active spermatozoa were produced at 89 hours; no further development occurred.

3. The addition of albumen to the yolk medium resulted in the production of active spermatozoa at 65 hours and the appearance of vitellaria and eggs at 88 hours; the eggs and vitellaria were abnormal.

4. The effect of adding various tissue extracts, reducing agents and casein hydrolysates to the basic yolk-albumen medium is described.

5. Vitellaria approaching 'normal' were observed when gelatine was added to the basic medium. Replacement of the gelatine with the amino acids - proline and hydroxyproline, gave similar results.

6. The findings are discussed with particular reference to the method of 'normal' egg-shell formation in Diplostomum phoxini.

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