CULTIVATION OF STRIGEID TREMATODES OF THE GENUS DIPLOSTOMUM

T H E S I S

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by

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C O N T E N T S

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LIST OF ABBREVIATIONS

C+M	Calgon and metasilicate cleaning solution.
G.T.	A modified Tyrode's solution, containing 1% glucose, and no bicarbonate.
G.P.T.	G.T., with phosphate buffer added.
H.S.	Horse serum.
Basic medium	(a) In Section IV; horse serum, egg albumen and G.P.T. (b) In Section V; yeast extract, horse serum and G.P.T. (c) In Section VI; yeast extract, albumen and G.P.T.
A.A.I. A.A.II.	Amino acid solutions referred to on page 76.

INTRODUCTION

The aim of this work was to investigate the physical and chemical conditions which promote the development of the metacercaria of Diplostomum phoxini (Strigeida, Trematoda) to the adult stage, and to apply some of the techniques used to other species of strigeid trematodes.

A parasite which is established in its host has little control over its environment, depending on its host for the maintenance of suitable physical conditions, and for the supply of food necessary for growth and development. It is possible, by direct measurement, to obtain a certain amount of information about physical conditions in the environment, such as temperature, and even pH and oxygen tension, but the chemical conditions are more difficult to determine. Many attempts have nevertheless been made to obtain more information.

The earliest work consisted of observing the effects on parasites of complete starvation of the host. Reid (1942) quoted five different reports of various types of intestinal worms which lost weight, showed decreased egg-production, or were expelled from their hosts, under these conditions. Reid (1940, 1942, 1944)

and Reid & Ackert (1941) showed that strobilae of the fowl cestode, Raillietina cesticillus, were expelled after 24 - 48 hours of starvation of the host, and that this loss of strobilae was associated with a reduction in the worms' glycogen content, to about one-eleventh of its normal level. This finding suggests that R. cesticillus is dependent on the carbohydrate in the host diet. In accordance with this Hymenolepis diminuta was found to be very sensitive even to partial restriction of carbohydrate (Chandler, 1943). There is much evidence, however, that for many other types of nutrient, parasitic worms are independent of their host's diet. When rat hosts were deprived of protein, H. diminuta actually grew to a larger size than worms in rats on full protein diets, and when vitamins A, D, E and B_1 were omitted from the diet, no adverse effects on the worms were produced (Chandler, 1943; Addis & Chandler, 1944, 1946). Chandler suggested that the worms absorb nitrogenous substances directly from the mucous membranes, but it is not known whether they can synthesise the vitamins themselves, or whether they obtain them from the mucosa, or from the intestinal flora. Clearly it is not easy to establish nutritional requirements when they may be contributed by all these

different sources.

A further complication in studies of this kind is illustrated by the work of Foster & Cort (1932, 1935) who reported that the hookworm, Ancylostoma caninum, showed increased egg-production in dogs fed on a diet which was deficient mainly in vitamins A and B, and in minerals. In this case it seems clear that the resistance of the host was depleted by the inadequate diet. Similar examples for other parasitic nematodes can be quoted. Vitamin A deficiency in the diets of rats caused decreased resistance to Nippostrongylus muris (Spindler, 1933) and to Trichinella spiralis (McCoy, 1934). These observations, though of interest in the understanding of host-parasite relationships, illustrate the complex conditions which make it difficult to investigate the nutritional requirements of helminths.

Von Bonsdorff (1956) investigated the position of Diphyllobothrium latum in the intestine of infected patients by withdrawing intestinal content samples through a tube inserted through the mouth. The presence of eggs in samples indicated the presence of tapeworms. He attempted to correlate this with vitamin B_{12} deficiency symptoms (i.e. pernicious anaemia) shown by the

patients, and concluded that the worms may absorb significant amounts of vitamin B_{12} from the contents of the proximal region of the intestine, since patients showed symptoms of deficiency of the vitamin, if eggs could be recovered within 130 cm. of the teeth. If the worms were situated further down the intestine, no deficiency symptoms were observed. Also, if small doses of filicin were given, worms situated proximally could be driven further down the intestine without being expelled. This cured the deficiency symptoms without removing the parasite.

Although it appears that the tapeworm was depriving the patients of vitamin B_{12} in the proximal region of the intestine, it is not known whether the tapeworm assimilates the vitamin or whether it destroys it. Certainly there is no simple equilibrium between the vitamin B_{12} concentration in the intestine, and that in the tapeworm. Von Bonsdorff added that this problem cannot be solved until Diphyllobothrium has been successfully cultivated in vitro.

Another method of investigating helminth nutrition, which is used by an increasing number of workers, is that of administering radioactive substances to the host,

and measuring the uptake of the labelled substances in the parasite. The results of four such experiments are considered here.

(1) Nyberg (1952, 1958) used Co^{60} - labelled vitamin B_{12} in an attempt to elucidate the vitamin B_{12} metabolism of the tapeworm D. latum. He measured vitamin B_{12} uptake by the worms, after oral doses to infected patients. The magnitude of the uptake seemed to depend on a variety of unknown factors, which so confused his results that he was unable to show clearly that high vitamin B_{12} activity in tapeworms accompanied deficiency symptoms in infected patients.

(2) Chandler et al. (1950) gave intraperitoneal injections of radioactive thiamine (vitamin B_1) to rats containing Hymenolepis diminuta, and obtained results which showed that the worms derived thiamine from the host tissues. The thiamine content of the worms was found to be independent of thiamine in the host diet.

(3) Read (1950a) administered trace amounts of radioactive phosphorus to rats by stomach tube, and measured the uptake of phosphorus by the intestinal mucosa, and by the tapeworm, H. diminuta, with which the

rats were infected. In the presence of glucose, the radio-phosphate was rapidly absorbed by the mucosa, and only very slowly by the tapeworm. This result might have been misinterpreted, had not radio-phosphates also been given in the absence of glucose, and it was shown that the rate of phosphate uptake was similar in the mucosa and in the tapeworm. Beyond relating this effect to phosphorylation of carbohydrate which occurs in the intestinal mucosa, Read offered no further explanations of the results.

(4) Hankes & Stoner (1958) noted that when mice infected with Trichinella larvae were fed labelled tyrosine and tryptophane (DL-tyrosine-2-C-14, and DL-tryptophane-2-C-14) the encysted larvae incorporated significant amounts of C^{14}. Although this demonstrates that substances can pass through the cyst wall, it is not certain what these substances are. In the tissues of the host the C^{14}-labelled amino acids may be altered before entering the cysts. The presence of C^{14} in the Trichinella larvae does not, therefore, indicate that the worms contain, or need to absorb the particular labelled amino acid fed in the experiment.

Some contributions to the study of cestode physiology

have been made by castrating the host. Addis (1946) showed that growth of H. diminuta was stunted in castrated rat hosts, but that administration of testosterone or progesterone to the host cancelled this effect. Some of his work was confirmed by Beck (1951, 1952) who showed that egg-production was also decreased in castrated rat hosts. The rate of transaminations was decreased not only in the tissues of castrated rats, but also in the tissues of the tapeworms which they harboured (Aldrich et al., 1954). Under the conditions of castration the muscles of the rats and of the tapeworms are less rich in protein, and there is increased deposi-:tion of fat in the tissues. These workers suggested that lack of hormonal secretion by the host causes decreased transaminase activity, which has an inhibitory effect on protein synthesis. This increases channelling of Krebs cycle constituents toward fat synthesis. They presumed that the tapeworm tissues, as well as the rat tissues, are subject to this hormonal effect.

Theoretically, some knowledge of the physiology of a parasite can be obtained by observing the effect of any alteration in its environment. Alterations in the environment have been brought about by transferring the parasite from its normal site to some other site in the

host tissues, but the changes produced in this way are inevitably very complex, and involve host tissue reactions (Goodchild, 1954, 1958).

It is evident that the *in vivo* techniques of feeding hosts dificient diets, administering radioactive substances, castrating hosts, and transferring parasites to other sites, yield interesting results; but all these techniques are subject to complications produced by interaction with the host.

However, the conditions which affect and control the life of the parasite could be much more easily studied if these conditions could be altered one by one, and the effect of each observed separately. If parasites can be maintained *in vitro*, a method is provided whereby controlled conditions can be produced.

This approach has been used to study the physiology of protozoa such as the parasitic amoebae, trypanosomes and rumen ciliates. Smyth (1946 *et seq.*) and Hopkins (1952) demonstrated and discussed the value *in vitro* cultivation for investigating the ways in which the gut environment controls maturation and development of cestodes. Culture techniques have also been worked out for certain parasitic nematodes (Glaser & Stoll, 1938;

Glaser, 1940; Weller, 1943; Weinstein & Jones, 1956; Silverman, 1959; and others).

This work parallels the use of tissue culture as a means of investigating cell physiology. Some of the techniques of tissue culture have been adapted to suit the culture of whole organisms, but there is a danger in attempting to follow these techniques too closely, since the natural environment of an intestinal parasite, for example, differs greatly from that of cells such as are used for tissue culture.

The aim of all work of this kind is to produce a medium which supports normal growth and development, in which physical conditions are optimum. Every constituent should be essential to the organism, and should be a known chemical substance of which the nutritional value and optimum concentration have been individually determined. Tissue culture work commenced over fifty years ago (Harrison, 1907), but a medium satisfying all these requirements has not yet been produced for animal cells, although some very successful synthetic media have recently been developed (e.g. White, 1949; Healy, Fisher & Parker, 1954).

A number of attempts have been made to cultivate the

larvae and adults of trematodes, but the recorded observations have been mainly confined to survival time, no information being given about the requirements for normal metabolism and development. Ferguson (1940) showed that the metacercaria of Posthodiplostomum minimum developed to maturity in vitro, but neither the eggs nor the spermatozoa produced were normal. The medium used was complex, and no assessment of the nutritional value of its constituents was made.

Robinson (1956a) described a method for maintaining Schistosoma mansoni in vitro. He showed that undiluted normal horse serum to which 0.1% glucose was added, provided a medium in which adult worms remained active for up to two months. Eggs were produced in these cultures, but the length of time that egg-laying continued in culture was not established. This technique was used to investigate some immunological problems, but no further steps were taken to produce a synthetic medium (Robinson, 1956b).

The metacercariae of the strigeid trematode, Diplostomum phoxini developed to maturity in a medium consisting of the yolk and albumen of hens' eggs (Bell, 1958). The eggs produced by the cultured flukes, however, had abnormal shells. The life history of this trematode

is outlined in figure 1, and is described in detail by Rees (1955, 1957). A diagram of the metacercaria is given in figure 2, and of the adult fluke in figure 3. The many advantages in using this species for cultivation experiments have been outlined by Bell & Hopkins (1956). There is now the further advantage that the developmental stages between the metacercaria and the adult have been described, in order to provide criteria for assessing the efficiency of a medium (Bell & Smyth, 1958).

Although the yolk + albumen medium described by Bell (1958) was useful for a number of the preliminary experiments presented here, it was realised that its value was limited. The yolk of an avian egg is an extremely complex material, which is very rich in a great variety of proteins, lipids, carbohydrates, vitamins and enzymes (Needham, 1931; Romanoff & Romanoff, 1949). Attempts to break it down into its individual constituents in order to find out which are important nutritionally, and which are not, would be very laborious, and perhaps altogether impracticable. It seemed, therefore, that even if further attempts to modify this complex medium were successful in bringing about normal development of the fluke, this would still shed but

little light on the nutritional requirements of these trematodes. In order to investigate this problem of the nutritional requirements of Diplostomum phoxini, it was decided to try to prepare a totally water-soluble medium, using chemically defined substances as far as possible, but failing these, to use complex substances to which various analytical procedures could be applied.

FIGURE 1

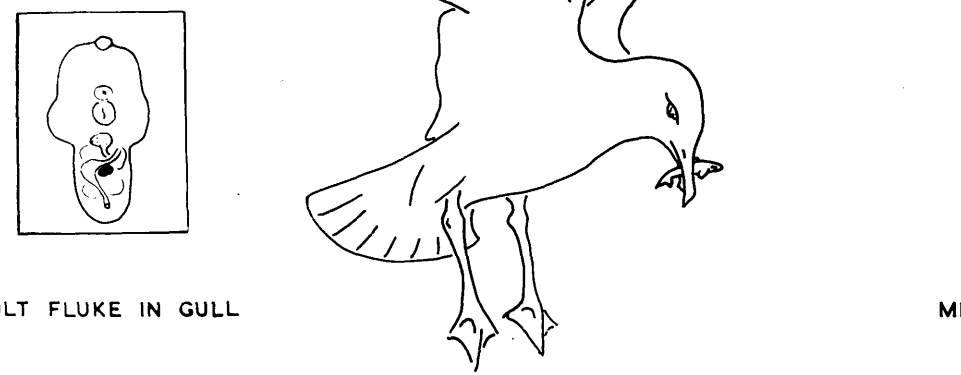
Diagram of the life history of Diplostomum phoxini

Typical host species:

(1) Snail; Lymnaea pereger (Müll).

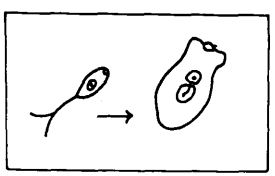
(2) Fish; Phoxinus phoxinus (L).

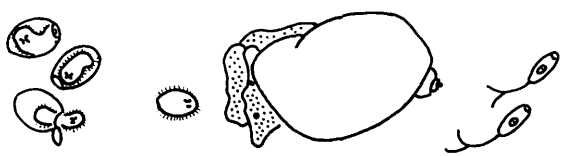
(3) Bird; Larus argentatus (Pontopp).



ADULT FLUKE IN GULL

MINNOW EATEN BY GULL





MIRACIDIA HATCH FROM EGGS IN GULL DROPPINGS

AND PENETRATE A SNAIL

WHERE THEY PRODUCE CERCARIAE, WHICH EMERGE,

AND ENTER A MINNOW.

THEY LOSE THEIR TAILS AND BECOME METACERCARIAE IN THE MINNOW'S BRAIN.

FIGURE 2

Diagram of the metacercaria of Diplostomum phoxini at X 400 magnification.

There is no protruding "tail", nor any rudiment of genitalia.

DIPLOSTOMUM PHOXINI METACERCARIA

0·2 mm.

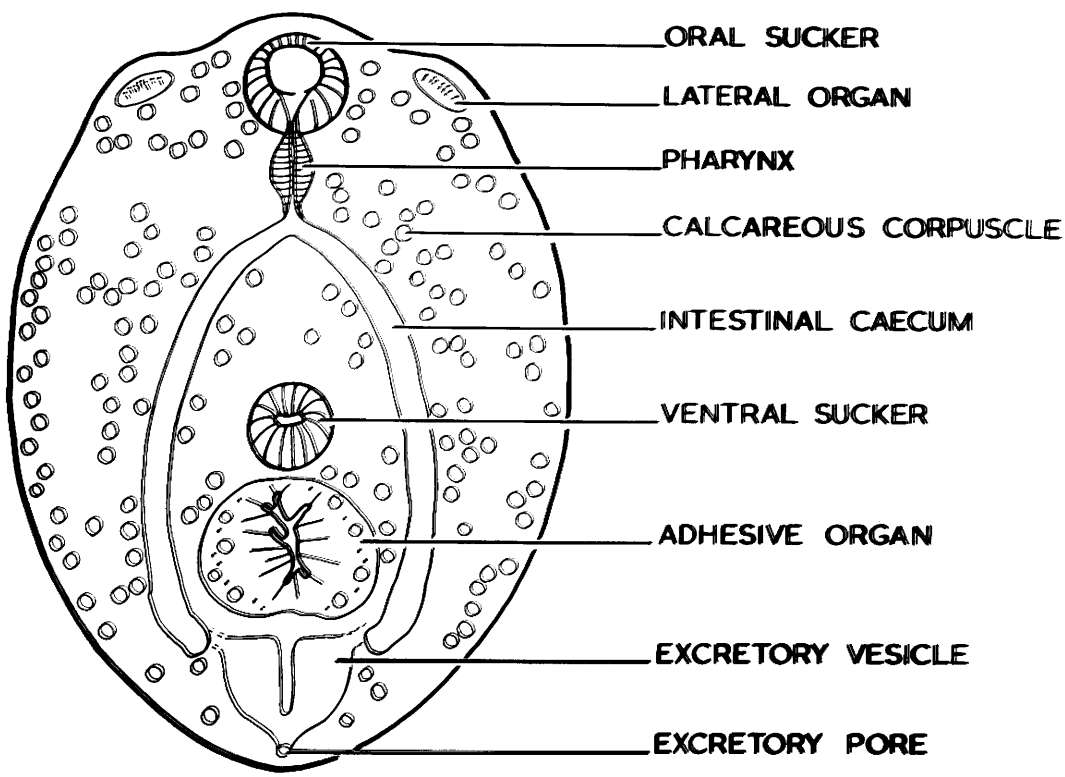
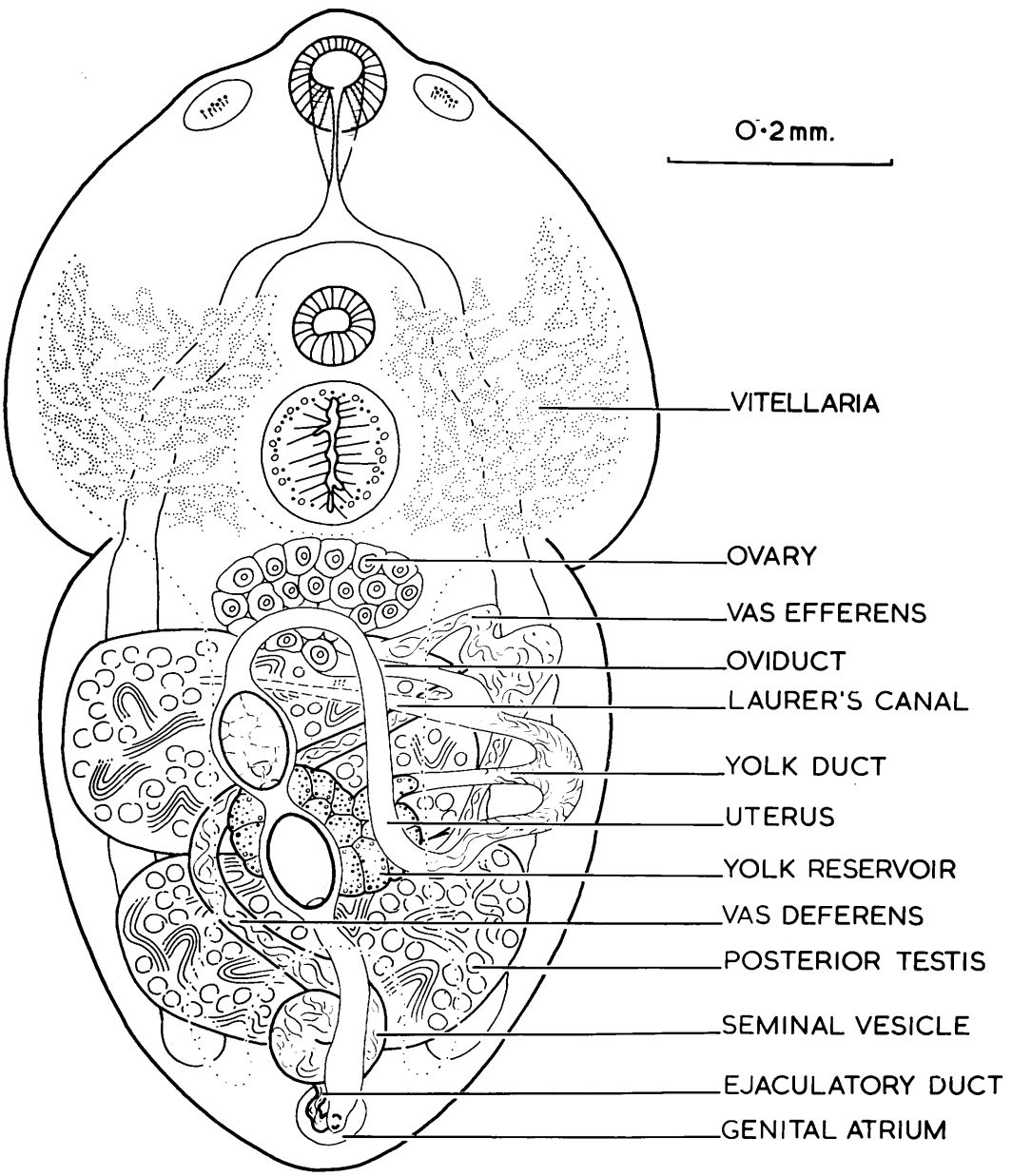


FIGURE 3

Diagram of the adult Diplostomum phoxini drawn from a living specimen at X 400 magnification.

This specimen matured in a duckling. A large "tail" region has developed, which contains most of the genitalia. The dotted line shows the area covered by the vitellaria.

DIPLOSTOMUM PHOXINI ADULT



MATERIALS AND METHODS

Aseptic Procedures

Aseptic precautions were observed throughout all culture preparations. Whenever possible these were carried out using a dispensing cabinet with a built-in ultra-violet source. This reduced the risk of contamination.

Bacterial contamination in media was detected by one, or all of the following:-

(a) microscopical examination of a sample of medium (oil immersion),

(b) examination of a smear of medium stained with methylene blue,

(c) in the case of transparent media, the appearance of cloudiness,

(d) significant changes in pH.

All contaminated cultures were discarded.

Cleaning of Glassware

Reagents used for cleaning glassware were Calgon

and sodium metasilicate, and the methods used were similar to those described by the Staff of the Tissue Culture Course (1955).

Procedure

(1) A stock solution of "C+M" was prepared:

360 g. sodium metasilicate

40 g. calgon

were dissolved in 5 litres of tap water.

(2) Glassware was rinsed to remove gross particles, placed in a stainless steel bucket, and covered with "C+M" solution diluted 100 times with tap water. This was boiled for at least 20 minutes.

(3) A long glass tube attached to a cold water tap was inserted into the bucket, and water was run in to rinse overnight.

(4) Glassware was transferred to a crate which was lowered into a bath of N/5HCl, where it remained for at least 4 hours.

(5) The crate was transferred to a pipette-washer, which rinsed the glassware for at least 4 hours.

(6) Glassware was dried at 100°C.

Special items

(1) Sintered glass filters were cleansed by passing through 300 ml. concentrated nitric acid, then two litres of tap water followed by 300 to 500 ml. of distilled water.

(2) Rubber liners from screw-capped containers and rubber stoppers, when new, were boiled in N/2 sodium hydroxide, rinsed with tap water, boiled in N/2 hydrochloric acid and rinsed thoroughly with tap water. This removed the sulphur "bloom" and other surface impurities. Thereafter these rubber articles were cleansed by boiling in tap water.

Sterilization of Glassware and other Items for Culture Preparations

(1) Petri dishes and crystallizing dishes covered with Petri dish lids were wrapped in paper.

(2) Pipettes were plugged with non-absorbent cotton wool, and the ends were either covered with paper or were placed in glass cylinders.

(3) Rubber stoppers were placed inside wrapped

crystallizing dishes.

(4) Other items such as filters, flasks and tubes were also suitably plugged or wrapped.

(5) The caps of screw-capped containers were slackly screwed in place.

(6) Dialysis tubing was fitted with a plugged glass tube at one end, closed with a double knot at the other, and inserted empty into containers three-quarters full of distilled water, following the method used by Fischer (1941).

All these items were sterilized by autoclaving at 10 to 15 lbs. pressure for 15 minutes.

Syringes and stainless steel instruments were sterilized in a boiling water sterilizer.

Preparation of Balanced Salt Solutions

Two modifications of Tyrode's solution were used. Analar chemicals were employed.

(1) Tyrode with extra glucose, but without bicarbonate (=G.T.). The following salts were used.

Sodium chloride (NaCl)	7.26 g.
Potassium chloride (KCl)	0.20 g.
Calcium chloride ($CaCl_2.6H_2O$)	0.40 g.
Magnesium chloride ($MgCl_2.6H_2O$)	0.10 g.
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	0.05 g.

10 g. glucose were dissolved in 40 ml. distilled water. This was added to the above salts dissolved in 960 ml. distilled water.

(2) G.T. with phosphate buffer (=G.P.T.).
The salts and glucose in (2) were used, but the sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) was increased to 0.18 g., and 0.30 g. of sodium phosphate (Na_2HPO_4) were added. To avoid precipitation of calcium phosphate the calcium chloride was not added until the majority of the water had been added.

Methods of Sterilizing Solutions

(1) Seitz-filtration. This was used for salt solutions and serum. 2-litre, 35-ml., or 10-ml. Seitz filters were used according to the volume of liquid to be sterilized. Positive pressure was used for all. The 10-ml. Seitz filter consisted of a hypodermic syringe with a "Swinney adaptor" outfit attached. The first and

last tenths of all filtered solutions were discarded.

(2) Sintered glass filtration. This was used for solutions of amino acids. Volumes up to 100 ml. were filtered, using negative pressure.

(3) Heat sterilization. Heat stable solutions such as glucose, yeast extract, acid and alkali were placed inside tightly sealed containers and either kept in a boiling water bath for at least 15 minutes, or autoclaved at 10 lbs. for 15 minutes.

Collection of Minnows

Minnows (Phoxinus phoxinus) were usually collected from the River Clyde, south of Hamilton. Baited traps and a small beam trawl net were employed. The fish were kept in running water tanks and fed on minced liver and dried Daphnia. In summer fish were not normally kept longer than one month before use, whereas in winter they were sometimes kept for longer than two months.

Collection of Metacercariae, and setting up of Cultures

Minnow brains were removed aseptically in the manner

described by Bell & Hopkins (1956). Metacercariae were liberated by teasing the brain tissue, and transferred by pipette to the culture medium. Accurate counting of the larvae was impracticable because of their small size, but most of the cultures contained 40 to 100 flukes.

Cultivation was usually carried out in 1 oz. universal containers, each containing 12 ml. of medium. Cultures prepared in this way were placed in a water bath at 40°C, in a shaker device which provided intermittent shaking (15 seconds on; 45 seconds off).

Measurement of pH of Culture Media

In general, before placing the flukes in the medium, the pH of a sample of medium was measured by a glass electrode, and recorded as the initial pH. Similarly at the end of the cultivation period the pH of the medium from each culture was recorded. At first the importance of measuring the pH immediately on opening the culture was not realised, but soon it was discovered that on standing, the pH of most culture media tended to rise slowly, probably due to the loss of carbon dioxide. The final pH was thereafter measured as soon as the

culture was opened, while the medium was still in the culture vessel.

Methods of Examining Cultures

Criteria

In evaluating culture media for helminths it is necessary to be able to state clearly what development has occurred. Criteria for assessing development were described by Bell & Smyth (1958). They divided development from the metacercaria to the adult into seven successive phases, carefully defining each phase. Although many of the criteria which they defined, have been found useful, there are two disadvantages in using these phases for measuring development:-

(1) The flukes do not all reach the same phase in any one medium. One culture, at the end of the period of incubation, may contain flukes at every developmental phase, from those with early genital primordia, to those which are almost completely mature. It is therefore impossible to state that in a certain medium, a certain developmental phase is reached.

(2) Flukes cultured *in vitro* do not always follow

the normal sequence of development, e.g. a yolk reservoir may appear in a fluke which still has an immature testis. According to the scheme proposed by Bell and Smyth, such a fluke has entered phase 6 before it has reached phase 5. Consequently it is impossible to say at what phase it is.

In the present work, when yolk media were used, in which a high degree of development was attained by most flukes, only criteria for assessing advanced development were employed. The numbers of flukes in the sample which showed (a) no yolk reservoir, (b) yolk reservoir but no eggs, and (c) eggs, were counted. This was easily done at X 40 magnification. When it was of particular interest to observe sperm production, several flukes were removed at random from the culture, and each was examined for the presence of sperm at a magnification of X 400.

In yeast and serum media, in which the flukes developed in a very variable fashion, the presence or absence of the following genitalia was used as a criterion for development:- (a) testes, (b) mature sperm, (c) active sperm, (d) ciliated uterus, (e) ovary, (f) vitellaria, (g) yolk reservoir, (h) eggs. The numbers of flukes in

the sample showing these genitalia were noted. In some sets of cultures the testes were described either as "early testes" (only the outlines visible) or "follicular testes" (more advanced stage in which round follicles are visible).

Sampling

Only small numbers of flukes were recovered from cultures in which yolk media were used; therefore it was possible to examine them all, using a microscope giving magnification of X 16 and X 40. Higher magnifications were sometimes used to check, for example, the presence or absence of a small yolk reservoir.

In cultures in which the medium was transparent, all the flukes could be recovered. Since the number of flukes in each culture was usually at least fifty, it was not practicable to examine every fluke in detail under a high power microscope. A method of sampling was necessary, and in all but a few preliminary experiments, certain well-defined methods were used.

Bell & Hopkins (1956) observed early development consisting of mitotic divisions in the tail region of Diplostomum phoxini in vitro. They counted the number of

dividing cells in 6 - 8 flukes from each culture, then averaged the four highest of these counts. Since very low counts were occasionally obtained in all of their media, they considered that an average of the highest counts would be of greater comparative significance than a mean of all counts.

A selective method of this sort was used for transparent media in which only a small number of flukes showed advanced development. The procedure was as follows:-

(1) The total number of flukes in the culture was counted.

(2) If the number of flukes in the culture was less than 50, a quarter of the flukes were selected using X 16 magnification. If there were more than 50, only 12 were selected, since it would have been too time-consuming to examine more than 12 in detail. Those selected were the ones showing the most advanced develop-:ment.

(3) The selected flukes were examined using high magnification (X 400). It was not easy to compare a culture from which 12 flukes had been examined, with one from which, for example, only 9 flukes had been examined. The results were therefore recorded as the proportion

out of 10 which developed the various genital structures. All the figures obtained for one medium were then totalled, and the average percentage calculated.

For example, if the results for the numbers of testes in three cultures were as follows:

Testes	No. of flukes examined
12	12
9	10
6	8

the figures recorded would be:

	Testes
	10
	9
	8
	27
Percentage	90

This method gave a measure of the most advanced development obtained in the media used, and allocated the same weight to each culture, regardless of the number of flukes it contained. Results for each culture are expressed as values out of ten rather than

percentages, which would have required the use of very large numbers, when in some cases, only a very few flukes were involved. When this method was used, the values obtained are referred to in the tables as the "proportion out of ten flukes developing genitalia".

Some inaccuracy was introduced when the number of flukes in the culture greatly exceeded fifty. In these cases only twelve flukes were selected, instead of one quarter of the total number, causing the development to appear better than it actually was. This occurred only in a minority of cultures, however, and in these cases results were interpreted with caution. Moreover, little danger of interpreting results wrongly was experienced as, for the most part, differences in development in the different media were quite distinct.

When cultivation conditions were improved, this selective method failed to give comparative information. A culture in which only 12 flukes out of 50 developed yolk reservoirs, appeared similar to a culture in which almost every fluke developed a yolk reservoir. Clearly another method of sampling was required, and so 25 living flukes were removed <u>at random</u> from the culture, and all of these were examined. A larger number could be examined than in earlier work, as no selection of flukes

was required, and as for the most part, development was better, and the presence of yolk reservoirs and large amounts of active sperm was easily and quickly observed. Also the presence or absence of the ovary was no longer used as a criterion, since it is an organ which is difficult to see, especially when poorly developed. Its importance in forming comparisons between cultures did not seem to justify the time spent in determining whether or not it was present.

This method was more satisfactory than the previous one, since it was much more quantitative, and did not involve the subjective and variable factor of selecting under a low power magnification what appeared to be the best flukes.

The result of developing suitable methods for recording experiments was that not all the results were directly comparable, and care had to be taken in attempting to compare results recorded using the selective method, with those recorded using the random method. However, all experiments of which the results were placed together in the same table, were carried out under the same conditions, and were recorded in the same way.

SECTION I: PHYSICAL CONDITIONS

Introduction

It is desirable in culture work to establish a set of physical conditions which is known to be suitable, before proceeding to extensive screening of media of varying chemical composition. This is not always practicable, however, since the chemical composition of a medium might be so unsuitable that it would be impossible to tell by examining the organisms, whether the physical conditions were harmful or otherwise.

In attempting to establish suitable physical conditions for the cultivation of D. phoxini, there was the advantage that previous workers had already established a medium (yolk + albumen) in which growth and development occurred, although little information was available about the physical conditions used.

1. pH

In nature the metacercariae of D. phoxini develop into adults in the small intestine of the herring gull,

and possibly, of other birds which feed on minnows. The pH of the proximal portion of the small intestine of most warm-blooded animals fluctuates as it receives acid stomach contents at intervals, but the strong buffering power of the bile and the digestive juices quickly brings the intestinal contents to a pH near neutrality, (Read, 1950b). The pH very near the mucosa is stable, and may differ slightly from the more fluctuating pH of the intestinal contents. Ball (1935), using a spear type of capillary glass electrode, obtained the following measurements from the small intestine of the rat:-

	Duodenal wall	Duodenal contents	Ileum wall	Ileum contents
pH (Average of at least two readings)	6.31	6.26	6.85	7.45

It was therefore expected that a pH near, or just below neutral would be suitable for the culture medium.

Bell (1958, fig. 10) showed that sperm production occurred in yolk medium, and that the addition of albumen not only accelerated and increased sperm production, but also allowed the development of vitellaria and yolk

reservoirs, and the production of abnormal eggs.

In the work presented here, these experiments were repeated. The pH of yolk medium, and of albumen were measured, using a glass electrode. The effect on the development of the flukes, of adjusting the yolk medium, and the yolk + albumen medium to a range of pH values, was observed. The effect of using different pH values for a medium consisting of yeast extract, horse serum, egg albumen, and balanced salt solution, was also observed.

Methods

Media containing yolk

(1) A fresh egg was painted all over its surface with an alcoholic solution of iodine. This was ignited.

(2) Sterile scissors were used to remove a part of the shell, about 1.5 cm. in diameter.

(3) The albumen was drained from the egg, through this aperture, into a sterile crystallizing dish.

(4) The aperture in the egg-shell was widened, and the yolk was inverted into another crystallizing

dish. Care was taken to remove albumen from the surface of the yolk, as completely as possible.

(5) Five millilitres of G.T. (Glucose-Tyrode) were added to the yolk, and blended with it.

(6) Ten millilitres of this yolk medium were placed in each culture container. If albumen was to be used, it was added separately in 2 ml. quantities to each culture.

This method was modified from the techniques used by Bell (1958).

Media containing yeast, serum and albumen

The preparation of serum + albumen medium is given below, on page 53. Water-soluble yeast extract was prepared and added, as described on page 60.

pH adjustments

The pH of the yolk, and of the albumen were altered by adding small quantities of 0.2 N sodium hydroxide and 0.2 N hydrochloric acid, respectively. Test preparations of yolk medium were prepared as follows, different egg yolks being used for each reading:-

3.0 ml. 0.2 N NaOH + 2.0 ml. G.T. + the whole yolk of an egg
2.0 ml. " " + 3.0 ml. " " " " " " " "
1.5 ml. " " + 3.5 ml. " " " " " " " "
1.0 ml. " " + 4.0 ml. " " " " " " " "
0.0 ml. " " + 5.0 ml. " " " " " " " "

The alkali was thoroughly mixed with the medium, and the pH of the resulting mixtures was measured. The values obtained in this way were used as a guide for the quantities of alkali which were required to produce the desired pH values in the culture media. It was necessary, however, to record the pH for each yolk that was used, as the yolks differed from each other in reaction.

Small quantities of 0.25 N hydrochloric acid were also added to 5 ml. of albumen, to discover what proportions were required to produce suitable pH values in the medium.

Adjustments in the pH of yeast + serum + albumen medium were made by small additions of 0.2 N sodium hydroxide or 0.2 N hydrochloric acid. By observing the effects of equivalent additions of acid or alkali to a sample of medium, the desired pH value was obtained.

Albumen is very alkaline (pH 9.0) and does not

usually dissolve completely in the medium, but leaves viscous masses which persist for some days, at the bottom of the culture containers. This caused a lack of homogeneity in the pH of the culture medium. It was found to be an advantage to make adjustments in pH by degrees, over a period of at least 15 hours. The medium was placed in the shaker apparatus between adjustments, to help it to equilibrate.

Results

After five days' incubation, flukes were recovered from cultures which had been set up to repeat and confirm the work of Bell (1958) on the yolk + albumen medium. Most of these flukes showed testes, ovary, ciliated uterus and vitellaria. Some showed active sperm, and some had large yolk reservoirs. About 20% had dark brown, unevenly tanned eggs in the uterus.

The pH values obtained for the test preparations of yolk medium are shown below:-

ml. 0.2 N NaOH	ml. G.T.	pH
3.0	2.0	8.5
2.0	3.0	7.3; 7.9
1.5	3.5	7.2; 7.7
1.0	4.0	7.1; 7.35
0.0	5.0	6.45; 6.6; 6.2

The following pH values were obtained when small amounts of acid were added to 5 ml. albumen:-

ml. 0.25 N HCl	pH
0.0	9.1
0.2	8.0
0.3	7.4
0.5	6.7
0.6	6.5

From these results it was possible to calculate the approximate amounts of acid and alkali required to alter the pH of yolk and yolk + albumen media (see Methods, page 33) to a suitable range of pH values.

Development of flukes in yolk and yolk + albumen media with adjusted pH is shown in Table I to IV. Table V shows the effect of pH in a medium consisting of yeast, serum, albumen and G.P.T.

Table I

The percentages of flukes showing advanced development after 5 days cultivation in yolk + albumen medium of various pH values.

Culture nos.	Initial pH of albumen	Initial pH of yolk + Tyrode (G.T.)	Final pH	Percentage occurrence of genitalia		No. of flukes examined
				Vitellaria and yolk reservoir %	Eggs %	
31B	6.5	5.7	5.9	0	0	10
27C 27C1	6.5	6.2	6.5	40	15	20
29C 52A 52A1	7.5	7.5	6.9-7.3	100	38	56
29D	8.0	7.8	7.2	80	33	15
31A 31A1	8.2	8.4	8.0	0	0	1*
27B	9.0	8.5	7.9	0	0	7*

*Only a small number of flukes were examined with high power magnification as all were poorly developed, and some dead.

Table II

The percentages of flukes showing advanced development in yolk medium of various pH values.

Culture nos.	Initial pH	Final pH	Percentage occurrence of genitalia		No. of flukes examined	Days incubated
			Vitellaria and yolk reservoir %	Eggs %		
32 1 4401	6.2	6.2	29	0	28*	6
44I1	6.8	6.7	100	30	37	6
44II1	7.3	7.0	100	54	24	6
44III1	7.9	7.3	0	0	8	6
44I2	6.8	6.7	100	48	23	7
44II2	7.3	7.0	100	50	20	7
44III2	7.9	7.3	36	5	22**	7

*Four of these were dead.

**About 30% of these showed no genitalia.

Table III

A comparison of the percentages of flukes showing advanced development in yolk medium with adjusted pH, and in yolk + albumen medium.

Culture nos.	Medium	Percentage occurrence of genitalia			No. of flukes examined	Days incubated
		Vitellaria %	Yolk reservoir %	Eggs %		
46 2c	yolk	25	5	0	20	4
46 2b	medium	100	38	25	24	5
44II1	(pH 7.0-	100	100	54	24	6
44II2	7.3)	100	100	50	20	7
20B	yolk	100	7	0	14	4
31C1 29A 27A 26A	+ albumen medium	100	82	36	56	5
28E1		100	92	42	12	6
27A1		100	90	50	10	7

Table IV

A comparison of sperm production in yolk medium with that in yolk + albumen medium.

Culture no.	Medium	Number of flukes		Days incubated	Final pH
		Examined	Showing active sperm		
32 1	yolk medium (pH 6.7 - 7.3)	6	1	6	6.2
44I1		5	0	6	6.7
44I2		6	1	7	6.6
44II1		4	0	6	7.0
44II2		5	0	7	6.8
46 2b		6	1	5	7.0
81A1		10	0	6	6.7
81B1		8	1	6	6.8
TOTAL		50	4		
PER-CENTAGE			8%		
29A1	yolk + albumen medium	6	2	5	6.6
47F1		8	2	4	6.9
47F2		6	1	6	6.9
77Y1		9	3	5	6.5
77Y2		11	4	6	6.7
TOTAL		40	12		
PER-CENTAGE			30%		

Table V

A comparison of the effects of pH 7.1 - 7.4 with that of pH 6.6 - 6.8 in yeast + serum + albumen medium.

Culture no.	Final pH	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs			
78B3	7.4	10	9	9	10	5	5	5	0	11	43	6
78B4	7.1	10	7	7	9	6	3	2	0	10	40	7
TOTAL (out of 20)		20	16	16	19	11	8	7	0			
PERCENTAGE		100	80	80	95	55	40	35	0			
78B	6.8	10	10	10	10	10	10	9	0	12	50	5
78B2	6.6	10	10	10	10	10	10	10	2	12	60	6
TOTAL (out of 20)		20	20	20	20	20	20	19	2			
PERCENTAGE		100	100	100	100	100	100	95	10			

Discussion

From the results shown in Table I it was concluded that of the pH values tested, an initial pH of 7.5 was the most suitable. As the pH often dropped considerably during the period of cultivation, it was not possible to ascertain a precise optimum value.

It was noticed that the amount of development in yolk + albumen medium which had been adjusted to pH 7.5, did not differ from that in yolk + albumen of which the pH had not been adjusted (see Table III). It was therefore concluded that when the alkaline albumen was mixed with the more acid yolk, a suitable pH was obtained.

Table II shows that development equal to that in yolk + albumen can occur in yolk alone, provided the pH is raised to a suitable level. The best results were obtained at pH 7.3 - 7.0.

Development in yolk medium of adjusted pH is compared with development in yolk + albumen, in Table III. The results show very little difference in development, except that it appears more rapid in the yolk + albumen medium. This effect shows itself so slightly that it may not be of any significance. Table IV, however, does show that albumen increases sperm production. The main effect of albumen, therefore, is to raise the pH of the yolk medium to a suitable level, but it seems probable that the protein

it provides is responsible for increasing the sperm production.

When these results were established, the pH of all media was adjusted to 7.0 - 7.3, until it was first noticed by the writer's colleagues that in medium consisting of horse serum, yeast, albumen and balanced salt solution, better development was obtained at a lower pH. Table V illustrates this point. Further experiments showed that good results could be obtained, at a final pH as low as 6.0 (see Culture No. 80A1, Section V, Table XX). These cultures did not differ greatly from those with a final pH value as high as 6.7 (see Culture No. 91D2, Table XX). It was therefore concluded that while the optimum pH for flukes cultured in yolk media was 7.0 to 7.3, it was more suitable to use a pH around 6.4 when the medium contained yeast, serum and albumen.

This observation may be similar to that made by Shaffer (1953) when culturing Endamoeba histolytica. In one medium consisting of horse serum, penicillin, and organisms of the genus Streptobacillus, the optimum pH for E. histolytica was 6.0. Excellent propagation of the amoebae occurred, however, in tissue bearing substrate in which the pH was initially 7.6, falling to 7.2 in 48 hours. Shaffer suggested that this effect was not due to the amoebae requiring a specific pH, but rather to certain enzymes in

the substrates, which were essential to the amoebae, but which varied in the pH at which they were effective. Certainly media containing living cells present very different conditions from yolk + albumen and yeast + serum + albumen media, but even the latter media contain different enzymes and other substances of which the activity may be affected by pH. For instance, albumen contains polypeptidases and dipeptidases of which the activity is increased if the pH of albumen is lowered to 8.0 for several days (Schültze, 1943). It also contains two erepsins, one with a pH optimum of 5.5, the other with a pH optimum of 7.0 to 8.0 (Van Manen & Rimington, 1935). The effectiveness of albumen at pH 7.6 might therefore be attributable to quite different factors from its effectiveness at pH 6.4.

Such considerations emphasize the complexity of media composed of natural products, and the desirability of a synthetic medium, even for the establishment of optimum physical conditions.

2. Osmotic Pressure

The osmotic pressure of a solution is given by its freezing point depression. The freezing point of yolk is -0.58°C to -0.60°C, and that of albumen is -0.45°C (Romanoff & Romanoff, 1949, page 420). The freezing point of Tyrode's solution is-0.62°C (Parker, 1950, page 77), which is close to that of yolk.

The effects of different osmotic pressures were not investigated but in non-yolk media the concentrations of substances were kept isotonic with Tyrode's solution, as far as was practicable. The flukes showed no visible osmotic disturbances in these media.

3. Temperature

The cultures were incubated at avian body temperature, which is 40°C. No attempts were made to determine the effect of abnormal temperatures.

4. Oxidation-Reduction Potential

Rogers (1949) provided the following measurements of oxygen tensions close to the mucosa, in mammalian small intestines. He used micro-electrodes devised by Davies & Brink (1942).

Sheep.....4-13 mm. Hg.

Rat.....8-30 mm. Hg.

These measurements show that an oxidizing environment exists close to the mucosa, but the oxidation-reduction potentials cannot be calculated from the oxygen tensions, without precise knowledge of all the oxidizing and reducing substances in the environment.

The oxidation-reduction potential of yolk is +300 mv. to +400 mv., while that of albumen is rather lower (Romanoff & Romanoff, 1949, page 412). 400 mv. is a relatively high potential, and may be too high for optimum conditions for the flukes. Some attempts were made to decrease the high potential of yolk by removing air by suction, and replacing it with oxygen-free nitrogen. Also the reducing substances, sodium dithionite and cysteine, were added. Measurements of the change in potential, after these procedures, were attempted, using a platinum electrode, but difficulty in achieving stable readings invalidated the results of the few cultures which were set up. Platinum can, under certain conditions, act as a catalyst of oxidation-reduction reactions, which may explain the variable readings. It was felt that provided the technical difficulties could be overcome, it would be important to investigate this subject further.

Summary

(1) The importance of determining optimum physical conditions for the cultivation of helminths, before carrying out extensive studies on nutrition, is discussed.

(2) Conditions of temperature, osmotic pressure, oxygen tension, and pH, in the cultivation of D. phoxini, are considered.

(3) Experiments designed to establish a suitable pH range for culturing D. phoxini are described.

(4) In yolk + albumen medium, the albumen was of value in raising the pH to a suitable level, besides having a purely nutritional effect which resulted in increased sperm production.

(5) In a medium consisting of yeast, serum and albumen, the optimum pH for development of flukes was rather lower than that observed in yolk + albumen medium. It is suggested that the optimum pH of complex media may depend on the optimum pH required for activity of the various enzymes present.

SECTION II: ROLLER TUBE CULTURES

Introduction

Bell's method of culturing trematodes in yolk + albumen medium, in universal containers placed in a water-bath, has the disadvantages that (a) the flukes cannot be observed without opening the cultures, and thus ending the period of cultivation, and (b) the small, transparent flukes are difficult to recover from the relatively large volume (12 ml.) of opaque, yolky medium.

It was therefore decided to make an attempt to culture the flukes using a roller tube technique. Roller tubes only contain up to 2.5 ml. of medium and the flukes can be observed sticking to the walls of the tubes, provided a strong light is used to show them up through the surrounding layer of medium. Bell & Hopkins (1956) used roller tubes when culturing D. phoxini in a medium consisting of duck serum and duck egg yolk. Under these conditions the flukes developed to the stage of producing a few active sperms. This encouraging result made it seem likely that in a more suitable medium, more advanced development would take place.

Since it was not known whether the use of these smaller volumes of medium would affect the flukes,

control cultures were set up, using similar small volumes in universal containers.

In roller tubes the medium has a large surface area in contact with the air in the culture tube. In order to prevent conditions being too aerobic, it was decided to flush some of the tubes with nitrogen before sealing them.

Method

Roller tubes (4½" x 1") were used, each containing 2 to 2½ ml. medium. Suba-seal stoppers were used to seal the tubes. These were rotated in an incubator at 39°C at a speed of 15 revs. per hour.

Six cultures were set up containing yolk medium with the pH adjusted to 7.35. Three of these were flushed with nitrogen containing 5% carbon dioxide before sealing.

Nine cultures containing yolk + albumen medium were set up. The ratio of yolk to albumen in three of these was 2 : 1, in another three, 1 : 1, and in the remaining three, 1 : 3. These cultures were also flushed with nitrogen before sealing.

Universal container cultures were set up in a similar manner to that described on page 32, except that the volume of yolk medium used per culture was varied.

Results

Yolk medium : Roller tube cultures

After three days the flukes were examined and some were found to have a few mature sperms. The cultures which had been flushed with nitrogen showed no improvement on the others.

Yolk + albumen medium : Roller tube cultures

A few flukes developed small yolk reservoirs by the sixth day, but vitelline development was poor compared with that in universal container cultures, and no eggs were produced.

Yolk + albumen medium : Universal container cultures

Table VI shows the results of the control universal container cultures. The values given for development are the percentage occurrence of vitellaria, yolk reservoirs and eggs, calculated on all the flukes recovered from the cultures.

Table VI

Development in different volumes of yolk + albumen medium in universal containers.

Culture no.	Volume of medium (ml.)	Ratio of yolk to albumen	Percentage occurrence of genitalia			No. of flukes examined
			Vitellaria %	Yolk reservoir %	Eggs %	
27D	2.0	1 : 1	97	44	0	32
26E1	3.5	1.5 : 2	94	29	6	17
27A	12.0	10 : 2	100	80	20	15
26A	12.0	10 : 2	100	73	32	22

Discussion

Under the conditions of this experiment vitelline development can occur in roller tube cultures, but the final stage of egg-production cannot be reached. A possible reason for this may be suggested. The flukes spend the majority of the time in culture, not immersed, but covered by only a very thin layer of the medium. This allows a rapid exchange of gases between the medium and the gas inside the tube. Roller tubes

were originally designed to provide an ample oxygen supply to cultured cells. The flukes normally live in an environment which contains little free gas, and certainly very little oxygen. Although the roller tubes were flushed with nitrogen, it was never possible to exclude all oxygen from the tube, as the mouth of the tube had to be exposed to air momentarily, before sealing.

In small volumes of medium in universal containers development is markedly depressed (Table VI). The increased exchange of gases, in particular of oxygen, between the gas in the culture tube and the medium, may well have been responsible for this. It might be thought that the increased ratio of albumen to yolk was responsible for the effect shown in Table VI, but this is not likely, as in the controls the albumen remains in a high concentration at the bottom of the culture tube, and does not mix evenly with the yolk.

Since the object in using roller tubes was to facilitate observation of the flukes, it became unnecessary to proceed further with this technique, when other experiments showed that development could occur in an almost transparent medium. When transparent media were used, there was no difficulty in recovering flukes from

the medium. Universal containers were otherwise very satisfactory, and so were used for all further cultures.

Summary

(1) Sperm and vitelline development occurred in flukes cultured in not more than 2.5 ml. of yolk + albumen medium, in roller tube cultures. The final stage of egg-production was not reached.

(2) Egg-production occurred in universal containers, in the same medium, provided a relatively large volume was used. Development was impaired when this volume was reduced.

(3) It is suggested that the importance of the larger volume was in minimizing gaseous exchange between medium and air in the culture vessel. Possible effects on the flukes of too much gaseous interchange are discussed.

SECTION III: REPLACEMENT OF YOLK AS A MEDIUM CONSTITUENT

Introduction

The failure of flukes to produce normal eggs in yolk + albumen medium showed either that some essential substances are absent, or that an unfavourable balance of conditions exists. Bell (1958) added a number of supplements to the yolk + albumen, in the hopes of supplying missing substances, and looked for effects on the egg-shell formation and the diazo reaction given by the vitellaria. The use of this colour reaction was described by Johri & Smyth (1956), and is important because it gives an indication of the presence of phenolic compounds in the vitelline cells, which are capable of being oxidized to form the material of the egg-shell. Abnormal colour reactions in diazo tests performed on the cultured flukes suggested abnormal or incomplete synthesis of egg-shell precursors. Bell noticed that the addition of the amino acids, proline and hydroxyproline to the yolk + albumen medium, produced a less abnormal diazo reaction in the vitellaria, but did not improve the egg-shell formation.

It was felt that addition of other nutritional

substances to the yolk might be informative.

Extracts of liver provide substances necessary for the culture of certain helminths (Dougherty, 1953; Stoll, 1953; Silverman, 1959). Yeast extract is well known as a valuable constituent of culture media for micro-organisms (Sykes, 1956). Liver and yeast extracts were therefore considered suitable substances with which to attempt to supplement the yolk + albumen medium.

All essential substances are provided by the fluke's natural environment, the host's gut. If there are any trace requirements missing in the yolk + albumen, an extract of the mucosa of a potential bird host might supply them. Among others, the pigeon is a suitable host for D. phoxini (Turnbull, unpublished) and so an extract of pigeon gut mucosa was chosen as a third substance to be added to the yolk + albumen medium.

At the same time it was hoped that some of these substances might be used to replace yolk. The many disadvantages in using yolk as a medium constituent have already been pointed out (page 11), and it seemed possible that substances such as liver and yeast extracts might provide suitable substitutes.

Methods

(1) Mouse liver extract

A freshly killed mouse was pinned on a board, ventral side uppermost, and this was placed inside the dispensing cabinet.

The ventral side of the mouse was painted with alcoholic iodine.

Using sterile instruments, the liver was removed and placed in a sterile 8 ml. screw-capped bottle.

One millilitre of glucose-Tyrode solution was added.

The liver and salt solution was homogenized by an overhead drive blender, rotating at speeds up to approximately 7000 r.p.m., for about half-an-hour. The homogenate was diluted to a volume of 10 ml. by addition of glucose-Tyrode solution and centrifuged at 270g for 10 minutes. The supernatant was removed by pipette and stored at $5^{\circ}C$.

(2) Autolysed yeast suspension

Autolysed yeast was obtained from Difco Laboratories. The dry powder was placed in sterile Petri dishes, in 0.3 g. quantities. Several millilitres of absolute

alcohol were added in order to sterilize the yeast. Yeast and alcohol were thoroughly mixed, and the Petri dishes with the lids off were placed inside the dispensing cabinet, directly under the lamps and the ultra-violet source for up to 15 hours. When the yeast was quite dry, 10 ml. of glucose-serum was added to each Petri dish, and as much of the yeast as possible was dissolved. The insoluble portion was kept as a suspension. This preparation was not stored, as it was prepared immediately before use.

(3) Pigeon mucosa extract

The gut of a pigeon which had been starved for about 18 hours was removed, and a section of small intestine 9 to 12 inches in length was cut out, starting 6 inches from the gizzard. This section was opened and the gut contents were gently brushed off the mucosa. The mucosa was scraped off the gut wall with a sharp scalpel. One to 2 ml. of mucosa was obtained. A little G.P.T. was added, and it was placed in a mortar with some fine washed sand, and ground thoroughly for 10 minutes. The homogenate was placed in a centrifuge tube, and the volume made up to 10 ml. with G.P.T. After 15 minutes centrifugation at 750g the supernatant was removed, diluted to a volume of 20 ml. with G.P.T.,

and Seitz-filtered. The filtrate was a clear, pale yellow liquid which was stored in sterile containers.

(4) Preparation of medium containing serum, G.T. (glucose-Tyrode) and egg albumen

Sterile Normal Horse Serum (No.2) was obtained in 250 ml. containers from Burroughs Wellcome, Ltd. 250 ml. of horse serum were mixed with 375 ml. of G.T. in a Matburn distributor. 8 ml. aliquots were dispensed from the distributor into universal containers.

Fresh egg albumen was collected as described on page 32; 2 ml. quantities were added to each universal container.

(5) Addition of liver, yeast and mucosa extracts

Liver extract was added in 1 ml. quantities to serum + albumen, and yolk + albumen medium. Autolysed yeast suspension was added in 2 ml. quantities to serum + albumen, and 1 ml. quantities to yolk + albumen. Mucosa extract was added in 1 ml. quantities to yolk + albumen. Cultures were set up in universal containers, in the usual way.

Results

Results from cultures in which mouse liver extract, autolysed yeast suspension, and pigeon mucosa extract were added in turn to yolk + albumen, are compared in Table VII with results from controls containing only yolk + albumen medium.

Results from cultures in which mouse liver extract and autolysed yeast suspension were added in turn to horse serum + albumen + G.T. are compared in Table VIII with results from controls containing only serum, albumen and G.T. In the yeast cultures a small number of flukes showed advanced development, although many were present which showed only early testes. Even the most well developed were smaller than flukes showing an equivalent amount of development in yolk + albumen medium.

Table VII

Development in yolk + albumen + G.T. to which mouse liver extract, autolysed yeast suspension, and pigeon mucosa extract, were added.

Culture no.	Type of extract added	Percentage occurrence of genitalia		No. of flukes examined	Days incubated
		Yolk reservoir	Eggs		
28 E2	Liver extract	100	45	11	5
28 E2a	"	100	50	8	5
29 B1	Autolysed yeast	71	41	17	5
77 Y1	Mucosa extract	100	22	9	5
77 Y2	"	100	55	11	6
28 E1	None (Controls)	100	42	12	5
29 A	None (Controls)	100	50	12	5

N.B. Eggs were all abnormal.

Table VIII

Development in horse serum + albumen + G.T., to which mouse liver extract and autolysed yeast suspension were added.

Culture no.	Type of extract added	Most advanced stage of development reached	Days incubated
28S4	None (control)	Extrusion of the tail	5
28S1	Liver extract	" " " "	5
28S2	"	" " " "	5
29E1	Autolysed yeast	Active sperm	4
30Y2	"	Active sperm, small yolk reservoir	5
33A1	"	Active sperm, yolk reservoir	5
33A2	"	Active sperm, yolk reservoir, darkly tanned vitelline material in uterus	6

Discussion

No effect was observed when the preparations of mouse liver extract, autolysed yeast suspension, and pigeon mucosa extract were added to the yolk + albumen medium, nor did the liver extract promote development, when added to serum + albumen medium. From this it was concluded that none of these preparations contained essential active substances which were not already present in the yolk and albumen, and the liver extract, even when added along with horse serum and albumen, did not contain enough nutrient substances to bring about development.

It was recognised that although liver, yeast and mucosa may all contain substances which can be utilized by the flukes, these substances could have been destroyed or altered during preparation of the extract, or alternatively, the extracting processes may not have been very efficient. In the case of mouse liver, and pigeon mucosa, richer supernatant solutions might have been produced if the homogenates had been allowed to stand over night before centrifuging. Only water was tried as an extracting solvent; organic solvents might be more successful in extracting important substances from liver and mucosa. There is also the possibility that some substances may not be active when freshly

derived from tissues, but may be activated by certain procedures. For instance, Dougherty (1953) showed that a certain fraction of liver protein became active as a nutrient for the nematode, Rhabditis briggsae, after being autoclaved, while another liver protein fraction became active when stored for two months.

Finally, the abnormalities which appear in flukes cultured in yolk media may not be due to the absence of essential substances at all, but to some adverse condition in the yolk.

At this stage in the work, there were, therefore, many problems which could have been investigated further, but results obtained using autolysed yeast, added to serum and albumen, were so interesting that it was decided to continue work with this medium. The autolysed yeast had a marked effect; active sperm were produced by several of the flukes, and a few showed some vitelline development. This yeast medium was much more trans-:parent than yolky media, and therefore had the practical advantage that there was little difficulty in recovering flukes from the cultures. The serum provided a good buffer, and the medium as a whole proved to be easier to separate into various fractions, for the purpose of determining which fractions contain active factors responsible for nutrition of the flukes.

Summary

(1) Addition of mouse liver extract, autolysed yeast and pigeon mucosa extract to yolk + albumen medium, failed to improve development. A completely abnormal egg was still formed by the flukes.

(2) Autolysed yeast was of considerable value as a nutrient when added to medium consisting of horse serum, albumen, and glucose-Tyrode. This produced a clear, well buffered medium which was therefore more suitable for carrying out studies on development.

(3) The possibility was suggested that abnormal egg formation in yolk + albumen medium may be due, not to the absence of an essential factor but to the presence of an adverse one in the yolk. It therefore seemed all the more desirable that a yolk-free medium should be developed.

SECTION IV: YEAST AS A NUTRIENT

Introduction

The discovery that yolk could be partially replaced by a yeast preparation encouraged the hope that addition of further substances to the yeast might make possible the elimination of yolk from the medium, without loss of development. The use of a yolk-free medium, which was easier to analyse than yolk-containing media, appeared to be a promising line of experimentation for investigations into the nutritional requirements of flukes.

As the yeast medium had been arrived at somewhat arbitrarily, no assessment of the individual nutritional value of each of the constituents had been made. It was essential to treat each constituent in turn, and experiments testing the nutritional value of yeast are described in this section.

Preliminary experiments were carried out using the suspension of autolysed yeast which has already been described, and also a serum-soluble extract of this preparation. The purpose of these experiments was to find (a) the most suitable way of preparing the yeast, and (b) the most suitable concentration to use.

Later an aqueous yeast extract was used, and two

concentrations of this were tested.

The nutritional value of the yeast was demonstrated by comparing basic media with and without yeast added.

It was thought probable that all the active substances in the yeast extract would be of relatively small molecular weight, since the process of autolysis, which is used in the preparation of yeast extract, involves break-down of large molecules. To check this point, the effect of using dialysed yeast was observed.

Materials and Methods

Basic medium

The basic medium in this section consisted, in each culture, of 2 ml. fresh albumen + 8 to 10 ml. 40% horse serum in G.T., or G.P.T.

Amino acids

The preparation of the amino acid solution used in this section is described on page 74, as "Method 1".

Autolysed yeast suspension

Autolysed yeast, in dry powder form, was placed in Petri dishes in 0.1, 0.3, 1.0 and 1.5 g. quantities.

Autolysed yeast suspensions were prepared from these as described earlier, on page 51, so that the concentrations of yeast in the culture medium were approximately 0.15%, 0.45%, 1.4% and 2.1% respectively.

Serum-soluble extract of autolysed yeast

When the serum-soluble extract was used, 10 ml. G.T. + H.S. was mixed with the dried, sterilized yeast, as before, and then the suspension was transferred to sterile centrifuge tubes and allowed to stand for one hour at 40°C in order to permit substances in the yeast to dissolve in the serum + salt solution mixture. The suspension was then centrifuged at 270 g for 30 minutes, and the supernatant used in 2 ml. quantities.

Water-soluble, heat-sterilized yeast extract

Yeast Extract was obtained from Difco Laboratories. Weighed quantities were dissolved in 100 ml. aliquots of distilled water and the solutions autoclaved. One to 2 ml. quantities were added to the basic medium in each culture.

Solutions of this yeast extract were prepared not more than a week before use, since during storage for lengthy periods a precipitate usually appeared, indicating some chemical change.

Dialysed yeast extract

The water-soluble yeast extract was dialysed in two ways. Visking Dialysis Tubing of one inch diameter was used. In experiment 60, a 4% yeast solution was dialysed inside a dialysing bag which was suspended in a one-litre beaker of distilled water. Dialysis was then continued for four days at room temperature, against distilled water which was changed twice per day. In experiments 63 and 72, the dialysing tube, and a concentrated yeast solution (16 g. in 25 ml. distilled water) were autoclaved separately. The yeast solution was introduced into the dialysing tube and dialysed for two days against running water at 15°C, then removed aseptically, made up to 100 ml. with half-strength G.P.T., and transferred to storage bottles. After dialysis yeast solutions had increased in volume by about one half, hence the value of completing the volume of the solution, on the termination of the dialysis procedure. The diluted balanced salt solution restored some of the lost salts without danger of over-compensating for the reduction in osmotic pressure. Yeast solutions became almost colourless, after being dialysed, showing that among other substances, the riboflavin had been extracted.

Results

In this section various different yeast preparations were added to the basic medium (=albumen + horse serum + G.P.T.).

The concentration of the autolysed yeast suspension used in experiments reported in the previous section was not accurately measured, but was about 0.45% yeast in the culture medium. In order to determine the most suitable concentration, cultures were set up containing a range of concentrations from 0.15% to 2.1% yeast in the medium. The results are shown in Table IX. Only a small number of the most well-developed flukes were examined from these cultures, and the Table gives the numbers of those flukes which developed genitalia.

Table IX

Development in various concentrations of autolysed yeast suspension in basic (=albumen + serum + G.P.T.).

Culture no.	Percentage autolysed yeast in culture medium	No. of flukes developing genitalia					Number of flukes examined (selected)	Days incub-:ated	Final pH
		Testes	Active sperm	Vitellaria	Yolk reservoir	Eggs			
43A1	0.15	6	3	3	3	0	6	6	8.0
43A2	0.15	2	0	0	0	0	2	7	7.9
43B1	0.45	5	4	2	0	0	5	6	7.6
43B2	0.45	4	4	2	2	0	4	7	7.7
43B3	0.45	3	3	1	1	0	3	7	7.7
47B2*	1.4	2	1	0	0	0	5	5	7.1
43C1**	2.1	0	0	0	0	0	12	6	7.0
43C2**	2.1	0	0	0	0	0	20	6	-

*Several dead.

**All dead.

Table X

Development in basic medium (albumen + serum + G.P.T.) and serum-soluble extract of autolysed yeast, compared with development in basic medium + heat-sterilized water-soluble yeast extract. (0.4% yeast used in both media.)

Culture no.	Type of yeast preparation	Method of sterilization of yeast	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Final pH
			Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitellaria	Yolk reservoir	Eggs				
53B1	serum extract of autolysed yeast	soaking with absolute alcohol	10	6	5	8	5	3	2	0	11	45	6	7.7
53B2			10	0	0	2	1	1	1	0	12	50	7	7.5
53C1	aqueous yeast extract	auto-claving	10	3	2	8	3	4	3	0	12	45	6	7.4
53C2			Not examined in detail. Three selected showed active sperm and yolk reservoirs. Others appeared well-developed								3	-	6	7.4

Table XI

A comparison of development effected by the two yeast preparations, (a) serum-soluble extract of autolysed yeast (b) heat-sterilized, water-soluble yeast extract, added to the basic medium + a solution of amino acids. (0.4% to 0.5% yeast used in these media.)

Culture no.	Type of yeast preparation	Method of sterilization of yeast	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Final pH
			Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitellaria	Yolk reservoir	Eggs				
48B1	serum extract of autolysed yeast	soaking with absolute alcohol	10	5	5	4	4	3	3	1	8	40	5	7.6
48B2			10	8	8	10	8	5	5	1	10	40	6	7.2
49A1			10	7	3	8	7	4	3	0	12	-	5	7.4
49A2			10	3	0	7	3	3	2	0	12	-	6	7.4
50A1			10	7	2	10	8	7	5	0	10	40	5	7.3
50A2			10	9	7	10	10	10	10	4	12	50	6	7.3
50A3			10	10	10	10	10	10	8	0	5	20	7	7.0
TOTAL (out of 70)			70	49	35	59	50	42	36	6				
PERCENTAGE			100	70	50	84	71	60	51	9				
57A1	aqueous yeast extract	auto-claving	10	8	3	10	8	5	3	0	12	60	5	7.2
57A2			10	8	8	9	9	9	9	0	12	70	6	7.2
68A2			10	10	10	10	10	7	7	0	10	42	6	7.2
69A1			10	6	4	10	6	0	0	0	8	30	5	7.3
69A2			10	9	9	10	10	10	8	0	11	45	7	7.0
70A1			10	8	8	10	10	8	8	1	12	65	6	7.2
70A2			10	5	5	5	4	3	3	0	8	35	6	7.2
70A3			10	5	4	6	4	2	2	0	10	40	6	7.2
TOTAL (out of 80)			80	59	51	70	61	44	40	1				
PERCENTAGE			100	74	64	88	76	55	50	1				

For the first time in this medium, a few flukes producing eggs were recorded (Table XI). These eggs were one to two in number in each fluke, and were abnormal in appearance, being similar to the eggs produced by flukes developing in yolk + albumen medium. Flukes producing active sperm were recorded in larger numbers than previously, and the amounts of sperm were greater than those produced in most of the earlier cultures, and very much greater than was observed in yolk or yolk + albumen cultures (see Table IV, Section I). In some flukes the sperm production appeared very similar to that observed in flukes matured in vivo.

Two different concentrations of the heat-sterilized water-soluble yeast extract were compared, since this extract seemed just as favourable as the serum-soluble preparation. The results are presented in Table XII.

Table XII

Development in the two different concentrations of yeast extract (water-soluble, heat-sterilized) added to basic medium + amino acid solution. (Basic medium = albumen + serum + G.P.T.)

Culture no.	Percentage yeast extract in medium	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incuba-:ted	Final pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
57A1	0.6%	10	8	3	10	8	5	3	0	12	60	5	7.2
57A2		10	8	8	9	9	9	9	0	12	70	6	7.2
TOTAL (out of 20)		20	16	11	19	17	14	12	0				
PERCENTAGE		100	80	55	95	85	70	60	0				
57C1	0.2%	10	8	8	9	8	8	6	0	12	100	6	7.2
57C2		10	8	8	10	9	6	6	0	12	100	6	7.1
TOTAL (out of 20)		20	16	16	19	17	14	12	0				
PERCENTAGE		100	80	80	95	85	70	60	0				

The effect of dialysing the aqueous yeast extract was investigated. Results from experiments using autoclaved dialysed yeast extract plus basic medium are shown in Table XIII. These are to be compared with control cultures in which the dialysed yeast was replaced by autoclaved whole yeast extract (Table XIV), and with "blank" cultures in which no form of yeast was added to the basic medium (Table XV). Results shown in these three tables are summarised, for ease of comparing them, in Table XVI.

0.5% yeast, either whole or dialysed, was generally used in these cultures, since the results in Table XII showed that concentrations of this order are suitable.

Table XIII

Development in dialysed aqueous yeast extract + basic medium. (Basic medium = albumen + serum + G.P.T.)

Culture no.	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incuba-:ted	Final pH
	Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reservoir	Eggs				
60B2	10	0	0	3	1	2	0	0	12	70	5	7.6
60B3	10	0	0	2	0	0	0	0	11	45	6	7.7
63B1	6	1	1	5	0	0	0	0	8	55	6	7.3
63B2	8	1	0	1	0	0	0	0	8	100	6	7.1
63B3	6	3	3	4	1	1	1	0	8	80	6	7.1
72D1	10	2	2	3	2	0	0	0	6	25	6	7.1
72D2	10	5	3	5	2	2	2	0	12	60	7	7.0
72D3	10	3	2	4	1	1	0	0	9	35	7	7.1
TOTAL (out of 80)	70	15	11	27	7	6	3	0				
PERCENTAGE	88	19	14	34	9	8	4	0				

Table XIV

Development in aqueous yeast extract + basic medium. (Basic medium = albumen + serum + G.P.T.)

Culture no.	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incuba-:ted	Final pH
	Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reservoir	Eggs				
53B1	10	6	5	8	5	3	2	0	11	45	6	7.7
53B2	10	0	0	2	1	1	1	0	12	50	7	7.5
59C2	10	4	4	8	4	4	4	0	9	35	7	7.6
60C2	10	2	2	8	3	3	3	0	12	50	6	7.5
60C3	10	3	3	7	5	3	3	0	12	70	7	7.4
62C1	10	2	1	7	3	2	1	0	12	50	6	7.3
62C2	10	4	4	10	7	7	4	0	12	60	7	7.3
62C3	10	3	3	10	3	3	2	0	12	60	7	7.3
64A1	10	4	3	10	4	3	1	0	12	60	7	7.3
65C1	10	2	2	3	2	0	0	0	12	50	6	7.4
67A1	10	6	4	9	6	1	1	0	7	28	5	7.2
67A2	8	4	4	3	2	0	0	0	9	35	6	-
67A3	10	6	5	6	5	1	1	0	8	32	7	7.5
TOTAL (out of 130)	128	46	40	91	50	31	23	0				
PERCENTAGE	98	35	30	70	38	24	14	0				

Table XV

Development in basic medium to which no form of yeast was added. (Basic medium = albumen + serum + G.P.T.)

Culture no.	Proportion out of 10 flukes developing genitalia									No. of flukes examined (selected)	No. in culture	Days incuba-:ted	Final pH
	Genital rudiment*	Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
63C1	4	5	0	0	0	0	0	0	0	12	65	6	7.3
63C2	4	3	0	0	0	0	0	0	0	11	45	6	7.4
63C3	0	4	0	0	0	0	0	0	0	12	70	6	7.4
71A1	2	2	0	0	1	0	0	0	0	9	35	5	7.3
71A2	3	7	0	0	0	0	0	0	0	12	50	6	7.1
71A3	3	7	0	0	0	0	0	0	0	12	50	7	7.3
TOTAL (out of 60)	16	28	0	0	1	0	0	0	0				
PERCENTAGE	28	49	0	0	2	0	0	0	0				

*No development other than a rudimentary plug of genital tissue in the extruded tail.

For ease of comparing the results of experiments in which the basic medium was supplemented with (a) whole yeast extract, (b) dialysed yeast extract, and (c) no yeast extract at all, the percentages calculated on totals from Tables XIII, XIV and XV are placed together in Table XVI.

Table XVI

A comparison of development of flukes in basic medium + (a) whole yeast extract (autoclaved), (b) dialysed yeast extract (autoclaved) and (c) no type of yeast supplement. (Basic medium = albumen + serum + G.P.T..)

Type of yeast additive	Percentage of those flukes examined which developed genitalia							
	Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitellaria	Yolk reservoir	Eggs
(a) whole yeast extract	98	35	30	70	38	24	14	0
(b) dialysed yeast extract	88	19	14	34	9	8	4	0
(c) no yeast extract	49	0	0	2	0	0	0	0

Discussion and Conclusions

High concentrations of yeast suspensions were harmful. Of the concentrations used, 0.45% and 0.15% were the most successful (Table XI). This may have been due to the flukes becoming buried in the layer of insoluble yeast sediment which collected at the bottom of the culture vessel to a depth of several millimetres in the highest concentrations of autolysed yeast. To eliminate any such effects, it was decided to remove the sediment by centrifugation. This resulted in a more uniform medium, and it was felt that more valid comparisons could be made when using such a medium. Consequently no further cultures using yeast suspension were set up.

No difference was detected between development in cultures in which the serum-soluble extract of autolysed yeast was used, and those in which water-soluble heat-sterilized yeast extract was used (Table X, XI). This shows that the essential nutrients in these yeast preparations were water-soluble and heat-stable.

Water-soluble yeast extract gives a clear yellow solution, and medium prepared from it was completely transparent. This greatly facilitated recovery of flukes after the period of cultivation. The slightest cloudiness indicated bacterial contamination, and cloudy

media could be discarded without delay. Contamination of autoclaved yeast extract medium was infrequent, whereas it was quite common with alcohol-sterilized autolysed yeast, undoubtedly because autoclaving is a more satisfactory method of sterilization.

0.2% and 0.6% autoclaved yeast extract were suitable concentrations to use (Table XII).

Development was depressed when yeast extract solution was dialysed before use (Table XIII). Sperm production was markedly reduced, and only very occasionally were yolk reservoirs or vitellaria observed. These cultures were nevertheless a distinct improvement on those in which yeast was omitted from the medium (Tables XV, XVI). This result was unexpected, since at first it was assumed that no high molecular weight substances, such as proteins would be present in yeast extract, as an analysis of autolysed brewer's yeast extract gave the following composition (Sykes, 1956):-

30 ± 3%	water
1%	vitamins
10%	inorganic salts
13%	carbohydrates
45%	free amino acids

A consideration of Sykes' description of the

preparation of yeast extract, however, showed that it is likely to contain a small amount of protein.

During the process of autolysis of yeast, the protein derivatives diffusing from the yeast cells in the first hour are mainly free amino acids. As the total nitrogen in solution increases, the relative formol titration drops, showing that a certain amount of protein, or at least, of high molecular weight break-down products of protein, enters the solution.

One would therefore expect dialysis of yeast extract solution to remove the inorganic salts, certain elements in the carbohydrate fraction, amino acids, purine and pyrimidine bases and those vitamins which are not bound to protein. Thus B vitamins such as riboflavin, thiamine, and folic acid will pass away through the dialysis membrane, since they are not protein-bound (Van Wagtendonk, 1955), whereas a certain amount of pyridoxine, which is a protein-bound vitamin in yeast (Robinson, 1951; page 298) would be retained.

Subsequent to the experiments using dialysed yeast, Williams et al. (in preparation) showed that yeast extract could very largely be replaced by pyridoxine. It therefore seems probable that pyridoxine is the active constituent left after yeast extract has been dialysed.

Summary

(1) The value of using yeast as a nutrient for the cultivation of D. phoxini was investigated.

(2) No decrease in development occurred when the insoluble portion of autolysed yeast was removed from the medium.

(3) Heat-sterilized, water-soluble yeast extract had all the activity of unheated autolysed yeast. In a medium containing heat-sterilized yeast extract, horse serum, egg albumen, balanced saline, and an amino acid solution, excellent sperm production occurred, and a few abnormal eggs were formed.

(4) 0.5% yeast extract in the medium was adopted as the concentration generally used, but 0.2% and 0.6% were also suitable.

(5) Dialysis of the yeast extract greatly reduced development, although a few flukes still produced active sperm and yolk reservoirs, showing that some essential nutrient remained in the undialysable fraction.

(6) The suggestion is made that pyridoxine may be the active constituent of this undialysable fraction.

SECTION V: HEN EGG ALBUMEN AS A NUTRIENT

Introduction

When egg albumen was used in the culture medium, it was hoped that the flukes would utilize it as a source of protein. In order to demonstrate its importance as a medium constituent, medium containing no albumen was compared with albumen-containing controls.

The protein composition of egg albumen is given by Fevold (1951). The total protein comprises 10 - 11% of the net weight of albumen. Six protein fractions have been recognised, namely:-

	Percentage of total protein in egg albumen
1) Ovalbumin	70
2) Conalbumin	9
3) Ovomucoid	13
4) Globulin	7
5) Ovomucin	2
6) Avidin	0.06

Ovomucoid is a glycoprotein, containing glucosamine and mannose. The globulin can be further

divided into three fractions. Since albumen contains not only a mixture of proteins, but also non-protein substances, it would simplify the medium greatly if the albumen could be replaced either by a single, purified protein, or by an amino acid solution. A solution of amino acids, derived from Tissue Culture Medium No. 703 (Healy et al., 1954) was therefore tested out as a substitute for albumen. Yeast extract was used in all cultures in this section and this solution included a number of amino acids which are present in yeast (see Table XVIII). The effect of a solution containing only those amino acids not present in the yeast (or present only in trace quantities) was therefore compared with the effect of the complete amino acid supplement based on Medium 703. Preparations of the proteins, casein, gelatin and bovine plasma albumin were also tried out, as possible substitutes for egg albumen.

Since egg albumen has a more complete amino acid composition than many proteins (although casein is also a good protein, as is shown in Table XVII), it was thought that if other proteins were used as substitutes, it might be necessary to augment these with amino acids. Also, since the flukes in nature live in an environment

in which much digested nutrient material is present, it is possible that they utilize free amino acids more easily than they do proteins. This would depend on the capability of the flukes' enzymes to hydrolyse the egg albumen proteins. The effect of the amino acid solution in the presence of albumen was therefore tested.

The protein, ovomucin, is the most insoluble of the albumen proteins. It is mainly responsible for the mucilaginous consistency of the albumen. When the medium contained dense, mucilaginous albumen, the flukes often became inextricably embedded in it. The development of these flukes was impaired. It was therefore of interest to remove the ovomucin, and test out the activity of the liquid fraction which remained.

In addition to proteins and carbohydrates, egg albumen also contains trace quantities of vitamins (Romanoff & Romanoff, 1949, pages 622-629). Dialysis of the albumen should remove these. This was carried out, and the dialysate tested for activity.

Table XVII

Amino acid composition of three proteins.

(% total protein; g. / 100 g. protein.)

Amino acids	Egg albumen (Fevold, 1951)	Casein (Cohn & Edsall, 1943)	Gelatin (Cohn & Edsall, 1943)
Glycine	3.2	0.5	25.5
Alanine	5.9	1.9	8.7
Serine	10.3	5.0	3.3
Threonine	4.1	3.5	1.4
Valine	7.1	7.9	0.0
Leucine	10.1	9.7	7.1
Isoleucine	7.7		
Proline	4.0	8.7	19.7
Hydroxyproline	0.0	0.2	14.4
Phenylalanine	7.8	3.9	1.4
Methionine	5.4	3.3	-
Cystine	0.5	0.4	0.2
Cysteine	1.3	-	-
Tryptophane	1.2	1.5	0.0
Tyrosine	3.9	5.4	0.0
Histidine	2.3	2.5	2.9
Arginine	5.9	3.7	8.7
Lysine	6.6	6.3	6.0
Aspartic acid	9.4	6.0	3.4
Glutamic acid	16.9	21.6	5.8
Total	113.6	92.0	108.5

N.B. Some of these estimations are not strictly accurate, since in two cases the totals exceed 100.

Table XVIII

The amino acids of yeast extract.

Amino acid	Present in yeast extract			Chosen for yeast supplement
	Source of information			
	Sykes (1956)	Pyke*	Difco*	
Glycine	+	+	+	
Alanine		+		
Serine				+
Threonine	+	+	+	
Valine	+	+	+	
Leucine	+	+	+	
Isoleucine	+	+	+	
Proline				+
Hydroxyproline				+
Phenylalanine	+	+	+	
Methionine	+	+	+	
Cystine	+	+		
Cysteine				+
Tryptophane	+		+	
Tyrosine	+	+	+	
Histidine	+	+	+	
Arginine	+	+	+	
Lysine	+		+	
Aspartic acid			+	+
Glutamic acid	+	+	+	
Glutamine		+		+
Glutathione				+

*Personal communications

Materials and Methods

Amino acid solution, based on Tissue Culture Medium No. 703 (Healy et al., 1954)

An amino acid solution was prepared and added to the culture medium in such a way that the concentrations of amino acids in the medium approximated those of Tissue Culture Medium No. 703. The amino acids were obtained from B.D.H., Ltd. Two methods were used.

Method 1. The following solutions were prepared:-

Solution I, Fraction A

	mg.
l - arginine monohydrochloride	35
l - histidine monohydrochloride	10
l - lysine monohydrochloride	35
dl - trytophane	10
dl - phenylalanine	25
dl - methionine	15
dl - serine	25
dl - threonine	30
dl - leucine	60
dl - isoleucine	20
dl - valine	25
dl - glutamic acid monohydrochloride	75
dl - aspartic acid	30
dl - α-alanine	25
l - proline	20
l - hydroxyproline	5
glycine	25
sodium acetate ($NaAc.3H_2O$)	47

These substances were dissolved in 80 ml. of Tyrode's solution without bicarbonate or glucose, heating to 80°C.

Solution I, Fraction B

	mg.
l - glutamine	50
cysteine hydrochloride	130
ascorbic acid	25
glutathione	5

These amino acids were added to Solution I, Fraction A, when it had cooled to room temperature.

Solution I was then made up to a volume of 240 ml. with 40% horse serum in G.P.T.

Solution II

	mg.
l - tyrosine	20
l - cystine	10

Tyrosine and cystine were dissolved in 50 ml. of 0.075 N HCl, heating to a temperature of not more than 60°C.

Ten millilitres of Solution II were combined with Solution I, making a volume of 250 ml. This was sterilized by Seitz-filtration, and stored in universal containers, in 5 ml. quantities. The addition of a further 5 ml. of amino-acid-free culture medium to each container produced concentrations of amino acids equal to those of Tissue Culture Medium No. 703, except in the cases of tyrosine and cystine, which were at one tenth of that concentration. This method of

incorporating the amino acids in the serum + G.P.T. had the disadvantage that if the medium was to be compared with another medium containing no horse serum, a separate amino acid solution had to be prepared. Only in experiments 48-59, therefore, was this method used. In experiment 60, and thereafter, amino acid solutions were prepared separate from other constituents of the culture medium.

Method 2. A solution having the constituents of Solution I described above, in concentrations ten times those of Tissue Culture Medium No. 703, was prepared in distilled water. It was sterilized by passage through a sintered glass filter, and stored in containers labelled "A.A.I.". 0.075 N HCl was used to dissolve l-cystine and l-tyrosine. This solution was prepared at concentrations twenty times those of Medium No. 703. It was passed through a sintered glass filter, and stored in containers labelled "A.A.II". 1 ml. of "A.A.I." + 0.5 ml. of "A.A.II.", when added to 10 ml. of medium gave approximately the concentrations of Tissue Culture Medium No. 703.

"Yeast supplement" amino acid solution

The following quantities of amino acids were dissolved in about 60 ml. distilled water, heating to

80°C:-

	g.
dl - serine	0.20
l - proline	0.20
l - hydroxyproline	0.04
dl - aspartic acid	0.20

The following were added to this solution when it was cool:-

	g.
cysteine hydrochloride	1.00
glutathione	0.04
l - glutamine	0.40

The solution was made up to a volume of 100 ml. with distilled water, and passed through a sintered glass filter. (N.B. Some of the cysteine hydrochloride did not dissolve, and was therefore removed by passage through a No.I Whatman filter paper before sterilization. The solid left on the filter paper weighed 0.1 g. after drying in a 40°C oven. It was therefore assumed that almost 90% of the cysteine hydrochloride had dissolved.)

Casein hydrolysate and casein

Casein hydrolysate (not vitamin-free) and light white soluble casein were procured from B.D.H., Ltd. A 1% solution of casein hydrolysate in distilled water was prepared and autoclaved. 1 ml. quantities were added to each culture. A 10% solution of casein in

distilled water was sterilized by boiling for two hours in a water-bath. This resulted in a rather cloudy solution.

Gelatin

Gelatin was obtained from Difco Laboratories. A 10% solution was prepared and added to the culture medium in the same way as for the casein. The gelatin formed a clear solution.

Bovine plasma albumin

Bovine Plasma Albumin (Fraction V) was supplied by Armour Laboratories. A 10% solution was Seitz-filtered and used in 2 ml. quantities in the cultures.

Homogenized, Seitz-filtered egg albumen

To 30 ml. of fresh egg albumen, 20 ml. G.P.T. were added. This was homogenized by a Nelco blender at a low speed for half-an-hour. It was then allowed to stand until most of the foam which had formed, subsided. The albumen preparation was then Seitz-filtered. 2 ml. quantities were used in each culture.

Dialysed albumen

Ten millilitres of fresh albumen were dialysed against running tap water for four days, under sterile conditions. During this procedure a white

precipitate formed, and the remaining liquid fraction was much less viscous than fresh albumen. Only this clear liquid fraction was used when 2 ml. quantities of the dialysed albumen were added to the medium in each culture.

Albumen dialysate

Albumen was dialysed by a modification of the method used by Harris (1952) for dialysing plasma, serum, and embryo extract. The assembly illustrated in figure 4 was used. The empty dialysis tubing was placed in position inside the flask, which contained 100 ml. of distilled water. The whole unit was then autoclaved.

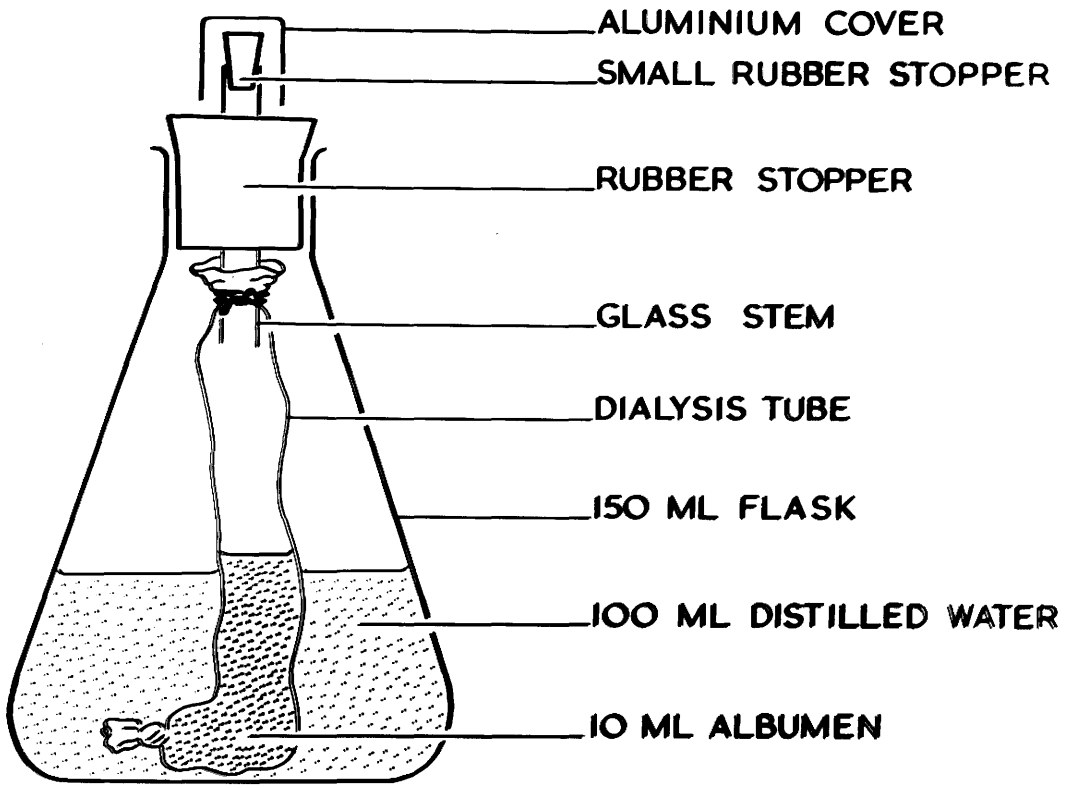
Ten millilitres of fresh egg albumen were introduced by pipette into the dialysing bag, through the glass stem. Every second day the dialysing bag was removed and exchanged for a freshly prepared one, containing 10 ml. of fresh albumen. Dialysis was continued for nine days.

The dialysate was reduced in volume from 100 ml. to 15 ml. in a vacuum-distillation apparatus, warming to 40°C. It was then passed through a hypodermic syringe, fitted with a "Swinney adaptor"

FIGURE 4

The assemblage used for preparing dialysate of albumen.

After sterilization of the whole unit, the albumen was introduced by pipette into the dialysis tubing.


ALUMINIUM COVER
SMALL RUBBER STOPPER
RUBBER STOPPER
GLASS STEM
DIALYSIS TUBE
150 ML FLASK
100 ML DISTILLED WATER
10 ML ALBUMEN

Seitz filter to ensure sterility.

One millilitre of albumen dialysate was used per culture.

Precipitation of ovomucin from egg albumen

Ovomucin was separated from egg albumen by Young (1937). The method consisted of diluting the albumen with two to three volumes of water at 0°C, at pH 6.4. An attempt was made to follow Young's method, although details of his procedure are lacking.

Axenic procedures were employed throughout.

Thirty millilitres of fresh egg albumen were placed in a crystallizing dish. 60 ml. of distilled water were added slowly, the albumen being stirred continuously. During this process precipitation of ovomucin commenced. The pH was lowered to 6.3 by additions of 0.5 ml. quantities of N/5HCl. This was also done slowly, with stirring. After each addition of acid, the pH of a test sample of the diluted albumen was measured. 2.5 ml. of acid were required altogether.

The diluted albumen was then stored at 5°C for two days, after which the precipitated ovomucin was removed by centrifugation, washed with water, and allowed to

re-dissolve in 12 ml. of 5% NaCl for one week. 1 ml. of the resulting gelatinous solution was used per culture. The remaining fraction was labelled "Fractionated Albumen".

Medium containing fractionated albumen was prepared, having the following constituents:-

	ml.
"Fractionated albumen"	50
4% yeast extract	12
Glucose salt solution (see below)	11
Horse serum	27
	100 ml.

This brought the concentrations of yeast extract and horse serum to nearly the same concentrations as those used in control cultures, i.e. 0.5% of yeast extract, and 27% of serum. This mixture was distributed into universal containers, in 12 ml. quantities.

The albumen was diluted to one third of its original strength, so that 16.6% (by volume) of the medium is contributed by the albumen fraction. The yeast extract contributed 12%, and the horse serum 27%, leaving approximately 44% of the volume, which required to be supplied by glucose Tyrode solution, in order to bring the concentrations of salts and glucose in the medium, to values similar to those of control media.

It was necessary, therefore, to incorporate in 11 ml. of distilled water, sufficient salts and glucose for 44 ml. of glucose Tyrode. The following amounts of salts were therefore dissolved in 11 ml., forming the glucose salt solution referred to above:-

	g.
Sodium chloride NaCl	0.319
Potassium chloride KCl	0.009
Calcium chloride $CaCl_2$	0.009
Magnesium chloride $MgCl_2$	0.004
Sodium dihydrogen phosphate NaH_2PO_4	0.002
Glucose	0.440

As has already been mentioned, 16.6% (by volume) of this medium is contributed by the albumen fraction. In control cultures 2 ml. of fresh albumen were added to 10 ml. of medium, so that the fresh albumen also constituted 16.6% (by volume) of the medium.

Results

The basic medium of this section consisted in each culture, of 1.5 ml. of 4% yeast extract (giving a final concentration of approximately 0.5%), added to 8 ml. of 40% horse serum in G.P.T.

Firstly, an experiment was set up to demonstrate the importance of egg albumen in the culture medium, and to compare its effect with that of an amino acid solution. Basic medium alone was used for control cultures. The results are shown in Table XIX.

Table XIX

Development in basic medium, in basic medium + albumen, and in basic medium + amino acids (Medium 703).
(Basic medium = yeast + serum + G.P.T.)

Culture no.	Additive to basic medium	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Final
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
90B1	none	10	0	0	3	1	0	0	0	12	60	5	7.0
91A1		6	0	0	1	0	0	0	0	7	30	6	6.8
91A2		5	0	0	0	0	0	0	0	8	32	6	6.9
TOTAL (out of 30)		21	0	0	4	1	0	0	0				
PERCENTAGE		70	0	0	14	3	0	0	0				
91F1	albumen	10	8	8	10	10	10	9	1	11	44	6	6.9
91F2		10	6	6	10	10	6	6	0	7	28	6	6.8
91F3		10	5	4	9	7	6	5	0	11	44	6	6.8
TOTAL (out of 30)		30	19	18	29	27	22	20	1				
PERCENTAGE		100	63	60	97	90	73	67	3				
91B1	amino	10	4	4	10	5	5	3	0	12	50	6	6.7
91B2	acids	10	6	6	8	6	5	5	0	10	40	6	6.9
91B3		10	4	3	6	4	3	3	0	10	40	6	6.8
TOTAL (out of 30)		30	14	13	24	15	13	11	0				
PERCENTAGE		100	47	43	80	50	43	37	0				

Since the results in Table XIX show that development is improved when basic medium is supplemented either with albumen, or with an amino acid solution, it seemed of interest to observe the effect of albumen and the amino acid solution added together to the basic medium. Results are shown in Table XX, together with results of albumen + basic medium cultures which were set up simultaneously for comparison. Media of pH values 6.0 - 6.7 were used for the cultures shown in Table XX, and the method of examining twenty-five flukes taken at random from the cultures, was used for recording the results.

Table XX

A comparison of development in albumen + basic medium, and in albumen + amino acids (Medium 703) + basic medium. (Basic medium = yeast + serum + G.P.T.)

Culture no.	Additive to basic medium	No. of flukes out of 25 taken at random, developing genitalia					Days incubated	Final pH
		Testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
83C1	albumen	25	12	14	8	0	6	6.5
83C2		25	21	20	18	0	6	6.5
83C3		25	19	9	6	0	7	6.7
91E2		25	17	10	7	0	6	6.6
TOTAL		100	69	53	39	0		
PERCENTAGE		100	69	53	39	0		
80A1	albumen + amino acids	25	19	20	17	1	6	6.0
80A2		25	17	20	17	1	7	6.1
80A3		25	15	17	12	0	7	6.3
91D1		25	23	14	9	1	6	6.5
91D2		25	21	18	17	2	6	6.7
TOTAL		125	95	89	72	5		
PERCENTAGE		100	76	71	58	4		

Results in Table XX show that development is improved when amino acids are added to basic medium + albumen. An attempt to substitute a hydrolysate of casein for the amino acid solution, is shown in Table XXI.

Table XXI

A comparison of the effects of an amino acid solution (Medium 703) and casein hydrolysate, when added to basic medium + albumen.

Culture no.	Additive to basic medium + albumen	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Final pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
70A1	amino acids	10	8	8	10	10	8	8	1	12	65	6	7.2
70A2		10	5	5	5	4	3	3	0	8	35	6	7.2
70A3		10	5	4	6	4	2	2	0	10	40	6	7.2
TOTAL (out of 30)		30	18	17	21	18	13	13	1				
PERCENTAGE		100	60	57	70	60	43	43	3				
54A1	casein hydroly-:sate	8	0	0	3	1	0	0	0	12	50	6	7.5
54A2		10	1	1	3	2	2	1	0	9	35	6	7.4
54A3		10	5	3	8	6	4	3	0	12	50	6	7.1
TOTAL (out of 30)		28	6	4	14	9	6	4	0				
PERCENTAGE		93	20	13	47	30	20	13	0				

Casein hydrolysate did not produce the same improvement in development as the amino acid solution of Tissue Culture Medium No. 703, when added to albumen + basic medium (Table XXI). Some investigation on the amino acid solution was therefore made, with a view to "pin-pointing" the important amino acids. The same amino acid solution was used, with and without its content of proline and hydroxyproline. Results are presented in Table XXII. (Cultures opened on the fourth day were not averaged, as development was not at a maximum.)

Table XXII

Development in the amino acid solution (Medium 703) with and without proline and hydroxyproline, added to basic medium + albumen. (Basic medium = yeast + serum + G.P.T.)

Culture no.	Amino acid solution used	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incu-:bated	Final pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
57A1	complete amino acid solution	10	8	3	10	8	5	3	0	12	60	5	7.2
57A2		10	8	8	9	9	9	9	0	12	70	6	7.2
57A3		10	2	2	3	2	2	2	0	12	29	4	7.5
TOTAL for A1,A2 (out of 20)		20	16	11	19	17	14	12	0				
PERCENTAGE		100	80	55	95	85	70	60	0				
57B1	without proline or hydroxy-:proline	10	8	8	8	6	6	3	0	12	40	5	7.2
57B2		10	8	7	10	8	6	6	1	12	45	6	7.2
57B3		10	5	4	3	1	1	0	0	12	45	4	7.6
TOTAL for B1,B2 (out of 20)		20	16	15	18	14	12	9	1				
PERCENTAGE		100	80	75	90	70	60	45	5				

No decrease in development was observed when proline and hydroxyproline were omitted from the amino acid solution, and so it was decided to try out an amino acid solution containing a greatly reduced range of amino acids. The amino acids of Tissue Culture Medium No. 703 include a number which are present in yeast extract. A solution containing only those amino acids not present in the yeast (or present only in trace quantities) was therefore used. These amino acids are referred to as "yeast-supplement" amino acids. Results of cultures comparing the effect of "yeast-supplement" amino acids and that of the complete amino acid solution (Medium 703) are shown in Table XXIII.

Table XXIII

Development in "yeast supplement" amino acid solution, added to basic medium + albumen, compared with development in control cultures containing the complete amino acid solution (Medium 703) and in "blank" cultures containing no amino acid supplement. (Basic medium = yeast + serum + G.P.T.)

Culture no.	Additive to basic medium	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Final pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitellaria	Yolk reservoir	Eggs				
74B1	yeast supplement amino acids + albumen	10	10	9	10	10	4	3	0	10	40	6	7.6
74B2		10	10	10	10	10	10	10	0	12	53	6	6.9
74B3		10	10	10	10	10	10	10	1	12	50	7	6.9
TOTAL (out of 30)		30	30	29	30	30	24	23	1				
PERCENTAGE		100	100	97	100	100	80	77	3				
74C1	amino acids (Medium 703) + albumen	10	10	10	10	10	3	3	0	12	48	6	7.1
74C2		10	10	10	10	10	6	6	0	9	36	6	7.0
74C3		10	8	8	9	4	4	4	0	9	35	7	7.0
TOTAL (out of 30)		30	28	28	29	24	13	13	0				
PERCENTAGE		100	93	93	97	80	43	43	0				
78B3	albumen only	10	9	9	10	5	5	5	0	11	43	6	7.4
78B4		10	7	7	9	6	3	2	0	10	40	7	7.1
TOTAL (out of 20)		20	16	16	19	11	8	7	0				
PERCENTAGE		100	80	80	95	55	40	35	0				

It was obvious that definite conclusions as to which amino acids are essential to the flukes, could not be drawn until more was known about their protein requirements. The beneficial effect of egg albumen suggested that it was utilized as a protein nutrient, but this was by no means established, as albumen contains other substances besides proteins. Attempts were therefore made to replace albumen with other protein preparations. Table XXIV shows the effect of substituting gelatin and casein solutions for albumen. 2 ml. of a 10% solution of each protein were added to each culture.

Flukes in gelatin cultures were very poor. When testes occurred they were early testes. Flukes in casein cultures were larger, less granulated, and some had follicular testes.

Table XXIV

A comparison of development in casein, gelatin and albumen, added to basic medium. (Basic medium = yeast + serum + G.P.T.)

Culture no.	Protein added to basic medium	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Final pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitellaria	Yolk reservoir	Eggs				
67C1	casein	4	0	0	2	0	0	0	0	9	37	5	7.3
67C2		7	0	0	0	0	0	0	0	12	48	6	7.5
67C3		3	0	0	2	0	0	0	0	6	25	7	-
TOTAL (out of 30)		14	0	0	4	0	0	0	0				
PERCENTAGE		47	0	0	13	0	0	0	0				
67G1	gelatin	6	0	0	0	0	0	0	0	5	20	5	7.0
67G2		3	0	0	0	0	0	0	0	10	41	6	7.4
67G3		2	0	0	0	0	0	0	0	6	25	7	-
72B1		6	0	0	0	0	0	0	0	5	20	6	6.9
72B3		7	0	0	2	0	0	0	0	10	40	7	7.1
TOTAL (out of 50)		24	0	0	2	0	0	0	0				
PERCENTAGE		48	0	0	4	0	0	0	0				
67A1	albumen	10	6	4	9	6	1	1	0	7	28	5	7.2
67A2		8	4	4	3	2	0	0	0	9	35	6	-
67A3		10	6	5	6	5	1	1	0	8	32	7	7.5
TOTAL (out of 30)		28	16	13	18	13	2	2	0				
PERCENTAGE		93	53	43	60	43	7	7	0				

Since casein gave slightly better results than gelatin, and since it had been shown that development improved in albumen-containing cultures when an amino acid solution was added, it seemed of some interest to observe how far development could proceed in cultures containing casein augmented with the amino acid solution of Tissue Culture Medium No. 703. Results are shown in Table XXV.

Table XXV

A comparison of development in basic medium to which was added (a) casein augmented with amino acid solution (Medium 703), (b) albumen augmented with the same amino acids and (c) the same amino acids, with no protein. (Basic medium = yeast + serum + G.P.T.)

Culture no.	Additive to basic medium	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incu-:bated	Final pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
69C1	casein + amino acids	3	0	0	0	0	0	0	0	8	31	5	7.0
69C3		10	1	1	3	1	0	0	0	12	51	7	7.2
70C1		10	1	1	4	0	0	0	0	10	40	6	7.2
TOTAL (out of 30)		23	2	2	7	1	0	0	0				
PERCENTAGE		77	7	7	23	3	0	0	0				
68A2	albumen + amino acids	10	10	10	10	10	7	7	0	10	42	6	7.0
69A1		10	6	4	10	6	0	0	0	8	30	5	7.3
69A2		10	8	8	10	10	10	8	0	12	45	7	7.0
70A1		10	8	8	10	10	8	8	1	12	70	6	7.2
70A2		10	5	5	5	4	3	3	0	8	35	6	7.2
70A3		10	5	4	6	4	2	2	0	10	40	6	7.2
TOTAL (out of 60)		60	42	39	51	44	30	28	1				
PERCENTAGE		100	70	65	85	70	50	50	1				
7001	amino acids only	10	2	2	5	3	3	3	0	12	83	6	7.1
7002		10	3	3	8	3	2	1	0	12	65	6	7.1
7003		10	4	3	8	3	2	1	0	12	55	6	7.2
TOTAL (out of 30)		30	9	8	21	9	7	5	0				
PERCENTAGE		100	30	27	70	30	23	17	0				

Casein gave very poor results, even when added with amino acids (Table XXV). Another protein, bovine plasma albumin was therefore tried. 2 ml. per culture of a 10% protein solution was again used. Results are presented in Table XXVI. Since the bovine albumin was sterilized by Seitz-filtration, two cultures were also set up, containing Seitz-filtered egg albumen. Fresh egg albumen cultures were used as controls too, and "blank" cultures, containing no albumen were also set up.

"Yeast supplement" amino acids and basic medium were used in all cultures.

Table XXVI

A comparison of development in (a) bovine plasma albumin, (b) fresh egg albumen, (c) Seitz-filtered albumen, and (d) no protein, added to basic medium + yeast supplement amino acids. (Basic medium = yeast + serum + G.P.T.)

Culture no.	Protein additive	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Final pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
76A1	bovine plasma albumin	10	3	0	4	0	0	0	0	12	50	6	6.8
76A2		10	0	0	1	0	0	0	0	12	140	6	6.5
76A3		10	2	2	3	0	1	0	0	12	120	7	6.5
TOTAL (out of 30)		30	5	2	8	0	1	0	0				
PERCENTAGE		100	17	7	27	0	3	0	0				
75C1	egg albumen	10	10	10	10	10	10	10	0	12	50	6	6.9
75C2		10	10	10	10	10	10	10	0	12	66	6	6.9
78C1		10	10	10	10	10	10	10	0	12	85	6	6.6
78C2		10	10	10	10	10	10	10	1	12	50	5	6.6
78C3		10	10	10	10	10	10	10	2	12	65	7	6.5
TOTAL (out of 50)		50	50	50	50	50	50	50	3				
PERCENTAGE		100	100	100	100	100	100	100	6				
78A2	Seitz-filtered egg albumen	10	10	10	10	10	10	10	3	12	84	6	6.3
78A3		10	10	10	10	10	10	10	1	12	90	7	6.3
TOTAL (out of 20)		20	20	20	20	20	20	20	4				
PERCENTAGE		100	100	100	100	100	100	100	20				
76B2	none	10	1	0	3	0	0	0	0	12	120	7	6.5
91C1		10	0	0	5	1	0	0	0	12	55	6	6.7
91C2		10	2	1	5	4	3	3	0	12	49	6	6.6
TOTAL (out of 30)		30	3	1	13	5	3	3	0				
PERCENTAGE		100	10	3	43	17	10	10	0				

Results in Tables XXIV to XXVI showed that egg albumen, whether in its fresh state, or after being passed through a filter, was nutritionally superior to casein, gelatin, and bovine plasma albumin. Two analytical procedures were therefore carried out on the egg albumen. The first was to dialyse the albumen, collect the dialysate and compare results from cultures containing (a) dialysed albumen, (b) albumen dialysate, (c) dialysed albumen plus the dialysate, added together, and (d) whole, fresh albumen; each being added to basic medium and amino acids (Medium 703). Results of these cultures are shown in Table XXVII.

The second procedure was to separate ovomucin from the albumen. Results of this experiment are shown in Table XXVIII.

Table XXVII

A comparison of results using (a) fresh egg albumen, (b) dialysed albumen, (c) dialysate of albumen, and (d) dialysed albumen + albumen dialysate combined, added to basic medium + amino acids (Medium 703).
(Basic medium = yeast + serum + G.P.T.)

Culture no.	Type of albumen	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Final pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
73C1	"whole" fresh albumen	10	3	0	10	8	9	5	0	8	34	6	6.9
73C2		10	2	2	10	9	9	5	0	11	43	7	6.9
a 73C3		10	9	9	10	10	8	6	0	8	32	7	7.0
74C1		10	10	10	10	10	3	3	0	12	48	6	7.1
74C2		10	10	10	10	10	6	6	0	9	36	6	7.0
74C3		10	8	8	9	4	4	4	0	9	35	7	7.0
TOTAL (out of 60)		60	42	39	59	51	39	29	0				
PERCENTAGE		100	70	65	98	85	65	48	0				
73B1	dialysed albumen	10	0	0	4	0	0	0	0	5	21	6	6.7
b 73B2		8	0	0	3	0	0	0	0	6	10	7	6.7
73B3		10	3	0	5	0	0	0	0	4	15	7	6.8
TOTAL (out of 30)		28	3	0	16	0	0	0	0				
PERCENTAGE		93	1	0	53	0	0	0	0				
c 73A1	dialysate of albumen	10	0	0	4	0	0	0	0	11	44	6	6.9
73A2		10	2	1	6	1	2	1	0	9	37	6	6.7
TOTAL (out of 20)		20	2	1	10	1	2	1	0				
PERCENTAGE		100	10	5	50	5	10	5	0				
74A1	dialysed albumen + dialysate of albumen	7	1	0	0	0	0	0	0	10	40	6	6.6
d 74A2		10	2	2	3	1	1	1	0	12	40	7	6.7
74A3		10	0	0	1	0	0	0	0	10	54	7	6.7
TOTAL (out of 30)		27	3	2	4	1	1	1	0				
PERCENTAGE		90	10	7	13	3	3	3	0				

Table XXVIII compares the effects of

(a) fresh egg albumen,

(b) albumen from which ovomucin had been removed,

(c) ovomucin,

(d) no albumen.

These were added to the basic medium.

Table XXVIII

A comparison of development in (a) fresh egg albumen, (b) albumen from which ovomucin had been removed, (c) ovomucin and (d) no albumen, added to the basic medium. (Basic medium = yeast + serum + G.P.T.)

Culture no.	Albumen fraction	No. of flukes out of 25 taken at random, developing genitalia								Days incubated	Final pH
		Early testes	Follicu-:lar testes	Mature sperm	Active sperm	Uterus	Vitel-:laria	Yolk reser-:voir	Eggs		
83C1	fresh "whole" albumen	0	25	16	12	20	14	8	0	6	6.5
83C2		0	25	22	21	21	20	18	0	6	6.5
83C3		0	25	20	19	21	9	6	0	7	6.7
TOTAL		0	75	58	52	62	43	32	0		
PERCENTAGE		0	100	77	69	83	57	43	0		
83A1	albumen from which ovomucin was removed	8	17	3	1	7	2	0	0	6	6.3
83A2		9	16	4	2	5	3	1	0	6	6.2
83A3		0	25	10	8	8	5	4	0	6	6.3
86B1		0	25	9	9	15	9	7	0	6	6.6
86B2		1	24	12	10	17	8	4	0	6	6.5
TOTAL		18	107	38	30	52	27	16	0		
PERCENTAGE		14	86	30	24	42	22	13	0		
86A1*	ovomucin	2	23	8	5	14	8	1	0	6	6.6
86A2**		-	-	-	-	-	-	-	-	-	-
PERCENTAGE		8	92	32	20	56	32	4	0		
83B	no albumen	14	11	2	2	7	4	3	0	6	6.6
89B1		10	13	4	4	6	3	2	0	6	6.4
TOTAL		24	24	6	6	13	7	5	0		
PERCENTAGE		48	48	12	12	26	14	10	0		

*Only 20 recovered, as flukes almost inseparable from the ovomucin

**Only 4 alive, therefore development not recorded.

Discussion

Table XIX indicates that development in a basic medium consisting of yeast extract, horse serum and G.P.T. was substantially increased by the addition of fresh egg albumen. Only a very low degree of development took place in its absence, no sperm or vitelline cells occurring in flukes in these cultures. When a solution of amino acids (of Medium 703) was substituted for albumen, development was better than in basic medium alone, a certain amount of sperm and vitelline development occurring. Still better development was obtained in albumen-containing cultures, suggesting that the amino acids partly, but not completely replaced the albumen, The amino acids, however, had a beneficial effect even in the presence of albumen (Table XX). This effect was a fairly slight one, for although percentages of the total number of flukes showing development of vitellaria and yolk reservoirs are considerably higher for the amino acid containing cultures, one of the four cultures in which there were no amino acids showed equally large numbers of yolk reservoirs (i.e. culture No. 83, C2). The presence of a few eggs in cultures with amino acids, and their absence in cultures with no amino acids, however, does seem to indicate a slight improvement attributable to the amino acids.

It therefore appears that the amino acids, and the

albumen, instead of being interchangeable, each have some separate effect, so that the best results can only be obtained in the presence of both.

Cultures containing casein hydrolysate instead of the amino acid solution were not so successful (Table XXI). Perhaps the balance of amino acids, or the presence of certain peptides in the casein hydrolysate solution were harmful.

No decrease in development was observed when "yeast supplement" amino acids were used instead of amino acids of Medium 703 (Table XXIII).

Amino acids are probably beneficial in the presence of albumen, because they provide readily available nitrogenous nutrients. It is possible that the amino acid solution contains more essential amino acids than are present in albumen, but it is also possible that the amino acids of albumen, being in the form of proteins, are not so readily available to the flukes. The value of albumen in the culture medium may be primarily in supplying non-nitrogenous nutrient.

There are thus several possible explanations as to why albumen is not successfully replaced by amino acids. Bell (1958) found that the amino acids, proline and hydroxyproline, appeared to cause the vitellaria in

cultured flukes to give a more normal diazo reaction - presumably an indication of more normal vitello-protein synthesis. Albumen contains no hydroxyproline (Fevold, 1951). It therefore seemed possible that albumen was deficient in an essential amino acid, which was supplied by the amino acid solution. However, when proline and hydroxyproline were omitted from the amino acid solution, no difference was observed in development (Table XXII). It seems more likely either that albumen is supplying essential non-protein substances, or that specific proteins in the albumen are beneficial. Also albumins are well known to have a detoxicating effect, and it was noticed that a surprisingly large number of dead flukes occurred in some non-albumen cultures. The beneficial effect of albumen may well be partly, if not completely, due to a detoxicating effect. Experiments in which purified proteins were substituted for egg albumen tended to confirm the hypothesis that the effect of albumen is not due to the mere presence of protein. Table XXIV shows control albumen cultures in which development was much superior to that in gelatin and casein cultures. The flukes in the casein cultures were slightly better than in the gelatin ones, and a slight improvement was observed when amino acids were added with casein, but even this amount of development did not equal that in control cultures containing no protein substitute for

the albumen (Table XXV). Similarly, cultures with bovine plasma albumin gave no better results than controls with no protein substitute (Table XXVI). Seitz-filtration of the albumen, on the other hand did not destroy its activity. This confirmed the validity of comparing fresh egg albumen with Seitz-filtered bovine albumin (Table XXVI).

Dialysis of samples of albumen had a marked effect. A white precipitate formed from the albumen, and collected at the bottom of the dialysing bag, leaving the remaining albumen as a watery liquid of a much less viscous consistency than the fresh material. Table XXVII shows that cultures containing (a) dialysed albumen, (b) albumen dialysate, and (c) the two combined, were poor compared with the cultures with "whole" fresh albumen, and in fact, were no better than would be expected with no albumen at all (cf. cultures 76B2, 91C1, 91C2, Table XXVI; 70 01-3, Table XXV). This treatment, therefore, must have altered the albumen so as to render it ineffective nutritionally. The physical change (i.e. liquifaction) which took place is not likely to be responsible for this effect, as the Seitz-filtered albumen (Table XXVI) was equally liquified, and yet was still active. It seems clear, therefore, that a chemical change occurred, leaving the albumen in-:active as a nutrient for the flukes. As the dialysate had no effect on development, there was no evidence that it contained essential substances such as vitamins.

The white precipitate which was formed during dialysis almost certainly consisted of ovomucin, which is the most insoluble protein in albumen. Table XXVIII presents results of cultures containing albumen from which the ovomucin was precipitated as efficiently as possible. Development was poorer than in controls with "whole" fresh albumen, but better than in cultures containing no albumen. Attempts to use the re-dispersed ovomucin were unsuccessful as the ovomucin formed a thick gelatinous lump from which flukes could scarcely be extricated. Results from only one culture were obtained, and so not much can be concluded, but again the flukes were much more poorly developed than in fresh albumen controls, although sperm production was slightly better than in cultures with no albumen.

It is not clear whether this loss of activity of the albumen is due to the removal of ovomucin, or to the effect of dilution with water on the other proteins, or to the exposure of albumen to the air for many days before use, as was necessary for these experiments.

Since no substance has yet been found which entirely replaces the albumen, it is most desirable to cast some light on the factors which are responsible for its activity. It is evident that it might be enlightening to carry out experiments using (a) "whole" albumen which has been exposed to the air for several days, (b) albumen diluted

with varying amounts of water, (c) albumen which has been further fractionated by precipitation of proteins with ammonium sulphate, and (d) partially or completely hydrolysed albumen. If, however, standing in the atmosphere, or dilution with water alters the activity, it would be very difficult to interpret fractionation experiments. Also, if relatively mild procedures such as dialysis and protein fractionation severely alter the activity, hydrolysis is likely to have a more drastic effect. Further experimentation, therefore, may meet with many technical difficulties, but results presented here may form a helpful basis for such work.

Summary

(1) Egg albumen is important in stimulating development in a medium consisting otherwise of yeast, horse serum, glucose and balanced saline.

(2) An amino acid solution partially replaces the effect of the albumen, but also has a nutritional effect even when albumen is present. The probable roles of these nitrogenous organic substances are discussed.

(3) Casein hydrolysate does not have the same effect as the solution of amino acids used.

(4) The omission of proline and hydroxyproline from the amino acid solution has no effect, and no difference in development was observed when the variety, but not the quantity of amino acids had been greatly reduced. It was very probable that the yeast extract was supplying the majority of amino acids not present in this reduced amino acid preparation.

(5) Gelatin, casein and bovine plasma albumin are all ineffective in replacing egg albumen.

(6) Dialysis of albumen, and precipitation of ovomucin by dilution with water, produce a change in the albumen which greatly reduces its value as a medium constituent.

The physical change which took place during these procedures, causing the albumen to lose its mucilaginous consistency, was thought to have no effect, since good development occurred in albumen which had been similarly liquified by passage through a Seitz-filter.

SECTION VI: SERUM AS A NUTRIENT

Introduction

The original intention of including horse serum in the medium, was to provide a well-buffered basic solution in which it was known that the flukes survived well (Bell & Hopkins, 1956). It was decided to demonstrate the effect of horse serum by omitting it from the medium, and comparing results with those from serum-containing controls.

Calf serum, since it comes from a young animal, is richer in growth-promoting substances than horse serum, and has no toxic effects such as are often demonstrated by tissue culture workers, using horse serum. Attempts were therefore made to substitute calf serum for horse serum.

Table XXX shows approximate concentrations of substances in human blood plasma. Table XXXI shows results of estimations of certain constituents of horse serum. These two tables provide some information as to what substances are present in mammalian serum, and in particular, in horse serum. Among the micro-constituents of serum, growth-promoting hormones, and perhaps some enzymes might be considered likely to have

a favourable effect on development of flukes. These, however, are mainly labile substances of rather large molecular weight, which are continually being renewed in the blood of living mammals. In serum which has been removed and stored for some time, many of these substances are probably destroyed. Other constituents of serum include substances of relatively low molecular weight, such as urea, amino acids, cholesterol and glucose, in low concentrations. Although it is possible that some of these might be important in the nutrition of flukes, it seems much more probable that the proteins, which constitute over 7% of horse serum, are responsible for any beneficial effects. The total protein content consists chiefly of 3.1 g./100 ml. of albumin and 4.5 g./100 ml. of globulin. Some investigations were therefore made on the effects of serum albumin and serum globulins.

A commercially obtained preparation of serum albumin was tested as a possible substitute for serum. Horse serum was also fractionated in the hopes of isolating effective protein fractions. This consisted of (a) dialysing the serum, and (b) separating certain of the proteins from the dialysed fraction, by an ammonium sulphate precipitation technique.

Jacquez & Barry (1951) applied similar fractionation techniques to human placental cord serum, which they used for promoting growth in fibroblasts. The use of the technique was successful in that they showed that the growth promoting factors were confined to the "euglobulin" fraction. Dougherty & Keith (1953) produced evidence that an active factor in human plasma, used as a constituent of a medium for the free-living nematode, Rhabditis briggsae, was confined to the globulin fractions, as was most probably associated with the β-globulins. It seemed of interest, therefore, to isolate the fractions (1) and (2) shown in Table XXIX, comprising the γ-globulins, and the α-, β- and muco-globulins, respectively, testing out both these isolated globulin fractions, and also the residues, for activity. The technique described by Cohn et al. (1940) was used to separate these fractions from the horse serum.

Table XXIX

	Ammonium sulphate concen-:tration. Moles/ L.	Saturation of ammonium sulphate	Type of protein precipitated	% of total protein precipi-:tated
(1)	1.39	0.34	γ-globulin	20
	1.64	0.40	α-, β- and γ-globulin	15
(2)	2.04	0.50	α-, β- and muco-globulin	14
	2.57	0.62	crystalline albumin	32
	2.80	0.68	crystalline albumin, haemo-cuprein, choline esterase, glyco-protein phospha-tase	14

Table XXX

The composition of human blood plasma.

(From Best & Taylor, 1950.)

	Percentage in plasma
Water	91-92
Proteins (Albumin, globulin and fibrinogen)	7
Inorganic salts	0.9
Other substances (listed below)	not more than 1.0

Urea
Uric acid
Xanthine
Hypoxanthine
Creatine
Creatinine
Ammonia
Amino acids

Neutral fats
Phospholipids
Cholesterol
Glucose
Hormones
Antibodies
Enzymes

Table XXXI

Some components of horse serum.

(From Spector, 1956.)

Total protein	7,600	mg.	per	100	ml.	of	serum
Albumin	3,100	"	"	"	"	"	"
Globulin	4,500	"	"	"	"	"	"
Cholesterol	77.0	"	"	"	"	"	"
Uric acid	0.9	"	"	"	"	"	blood
Urea nitrogen	10-20	"	"	"	"	"	"
Creatinine	1.2-1.9	"	"	"	"	"	"
Lactic acid	10-16	"	"	"	"	"	"
Glucose	73.0	"	"	"	"	"	"

Materials and Methods

Normal Horse Serum (no. 2) was obtained from Burroughs Wellcome, Calf Serum from Oxo, and Bovine Plasma Albumin from Armour Laboratories.

Dialysis

One hundred millilitres of horse serum were dialysed against several changes of distilled water in earlier experiments, but later against running tap water at 15°C, for up to four days, using aseptic precautions. On completion of dialysis, the volume of serum was measured, and usually was found to have increased by about one half. The volume was then increased further to 300 ml. by addition of a salt solution which contained the appropriate amounts of salts for 300 ml. of G.P.T. This mixture was Seitz-filtered.

Separation of serum proteins

γ-globulins and α-, β- and muco-globulins were separated from horse serum by a method based on the Theorell technique, which was applied to the proteins of horse serum, by McMeekin (1939) and Cohn et al. (1940). These workers did not use sterile procedures, but were concerned with obtaining the purest proteins possible. For this work it was essential to take aseptic precautions, and it was more important to concentrate on obtaining

active fractions, rather than on purifying the separated proteins.

(1) γ-globulin

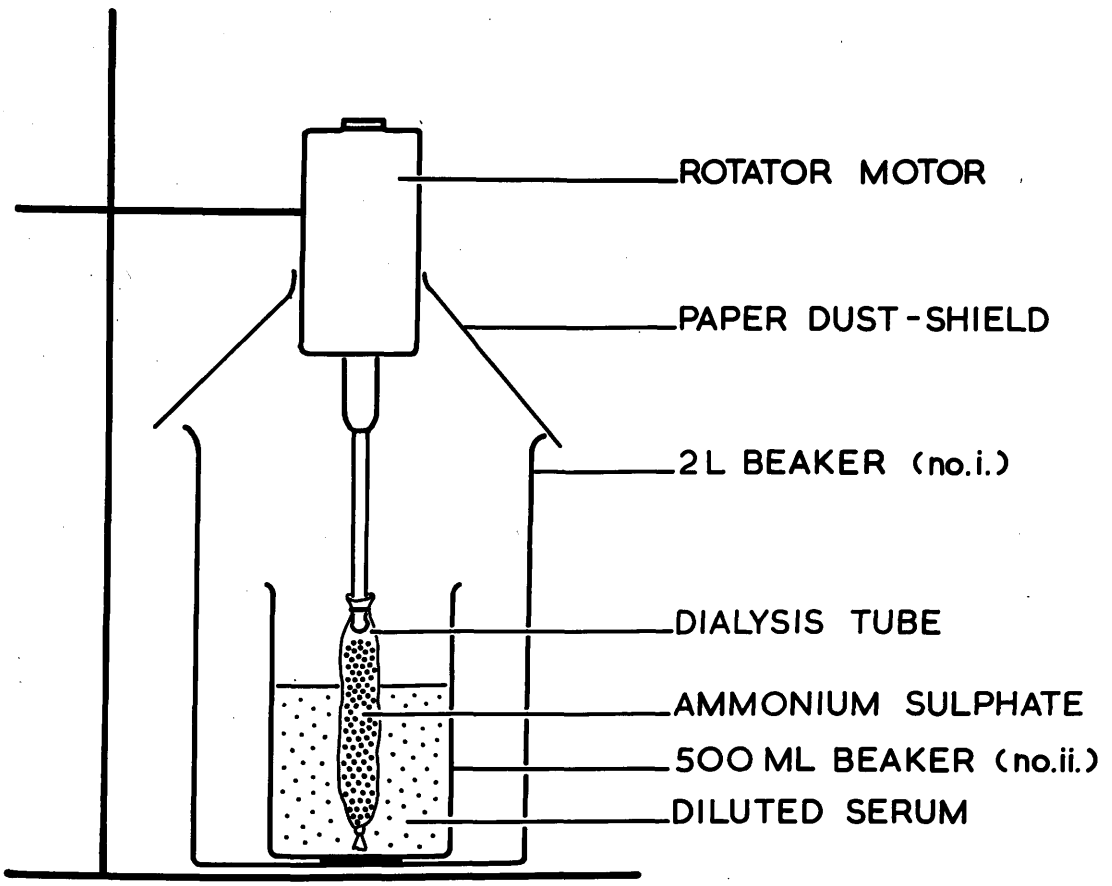
Two beakers (i, ii) were sterilized, one within the other, as in figure 5. 27.52 g. of solid ammonium sulphate were packed into a sterile dialysing bag (1 inch diameter Visking) by means of a glass tube fitted with a plunger (see figure 6). This was found to be the best way of filling the dialysing bag compactly, without contaminating it.

The dialysing bag was then attached to a rotating motor, and immersed in 75 ml. of horse serum which had been diluted with 75 ml. of distilled water, and placed in beaker no.ii (figure 5). The dialysing bag was allowed to rotate at low speeds for 44 hours at room temperature. During this period fluid from the serum entered the dialysing bag and ammonium sulphate became dissolved and passed through the membrane into the serum until an equilibrium was reached. The pH of the diluted serum was at first just above 7.0, but after addition of ammonium sulphate it had fallen about 0.7 of a pH unit. The final concentration of ammonium sulphate, which gave a 34% saturated solution, caused a fine, granular precipitate of γ-globulin to separate from the serum. Attempts were made to remove this

FIGURE 5

A diagram of the apparatus used for separating globulin fractions from horse serum, by preci-:pitation with ammonium sulphate.

Rotation of the dialysing bag, containing the solid ammonium sulphate, allowed the salt to be gradually and evenly dissolved in the serum.


ROTATOR MOTOR
PAPER DUST-SHIELD
2L BEAKER (no.i.)
DIALYSIS TUBE
AMMONIUM SULPHATE
500 ML BEAKER (no.ii.)
DILUTED SERUM

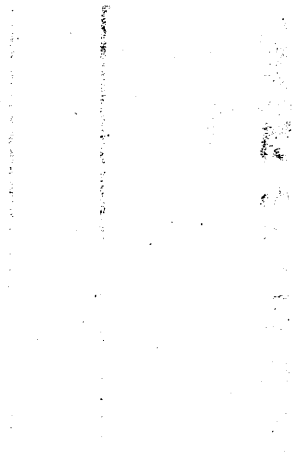
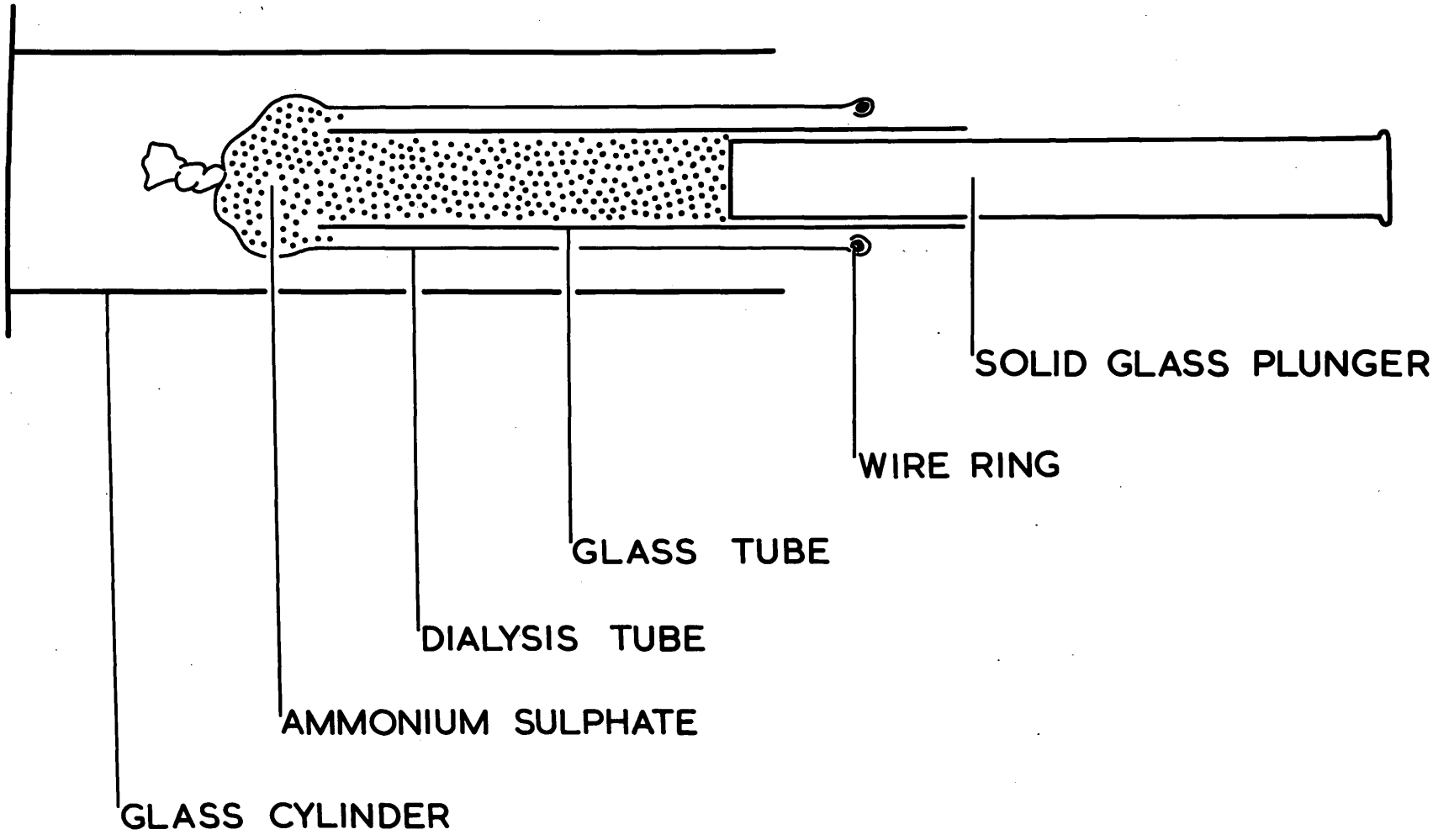


FIGURE 6

An illustration of the method used to fill the dialysing bag compactly with solid ammonium sulphate, in preparation for precipitation of serum proteins.

The dialysing bag was first sterilized by autoclaving inside the outer glass cylinder. The weighed salt was placed in the glass tube, and forced into the dialysing bag with the solid glass plunger. The outer glass cylinder served to prevent the outer surface of the bag becoming contaminated.


SOLID GLASS PLUNGER
WIRE RING
GLASS TUBE
DIALYSIS TUBE
AMMONIUM SULPHATE
GLASS CYLINDER

precipitate by passage through No.5 Whatman filter-paper, but this proved too coarse. A particularly fine grade of filter-paper was recommended, namely No.575 Schleicher and Schull, but this was not available, and so centrifugation was employed (1,685 g) to separate the precipitate from the supernatant fluid.

Precipitate

This was washed three times with 34% saturated ammonium sulphate solution, centrifuging between each washing to collect the precipitate and remove the ammonium sulphate washing. The precipitate was then dissolved in 35 ml. of distilled water, and dialysed against running tap water for about 24 hours during which time some of the globulins were re-precipitated. Dialysis was then continued for 24 hours against two changes of 0.9% sodium chloride (which caused the precipitated globulins to re-dissolve) and then against two changes of G.P.T., for 24 hours.

The γ-globulin solution was removed from the dialysing bag, diluted to a volume of 60 ml. with G.P.T. and passed through an asbestos clarifying filter.

Before use as a culture medium constituent, the

γ-globulin solution was diluted with G.P.T. to a volume equal to 2.5 times the volume of the original serum, so that the concentration of γ-globulin equalled that in G.P.T. containing 40% horse serum, which was used in control serum cultures.

Supernatant

The supernatant was dialysed against running tap water, then against changes of 0.9% sodium chloride, followed by G.P.T., in the same way as for the γ-globulin solution. The volume was measured before Seitz-filtration, and found to be almost exactly 2.5 times the original volume of the serum.

(2) α-, β- and muco-globulins

Separation of the α-, β- and muco-globulin fraction was carried out in exactly the same way, except that 50% saturated ammonium sulphate was used. 54.3 g. of solid ammonium sulphate was dialysed into 200 ml. of serum which had been diluted with distilled water to twice its original volume. A difficulty arose when it was discovered that in spite of aseptic precautions, the separated globulins contained a bacterial contamination. Seitz-filtration of a small sample showed that the globulins, being present as very

large molecules in colloidal solution, could not pass through the filter. This was evident as the globulin solution was of an opalescent appearance, typical of colloidal solutions, whereas the filtrate was completely colourless and transparent. It was therefore necessary to add antibiotics. To 100 ml. of the globulin solution, 20,000 units of penicillin, and 20,000 units of streptomycin were added.

The media prepared, incorporating this protein fraction, remained clear, and microscopical examinations at the end of the cultivation period showed no bacteria.

Results

The basic medium in each culture in this section consisted of 2 ml. of fresh egg albumen, plus 1½ ml. of 4% autoclaved yeast extract, plus 4.8 ml. of G.P.T. When serum was added to the medium, 8 ml. of 40% serum in G.P.T. was added in each culture to the albumen and yeast.

Table XXXII shows development in a medium which contained no serum but consisted of basic medium in which the volume of G.P.T. was increased to 8 ml. A few flukes with sperm and ciliated uterus occurred,

but a large majority showed only early testes. In cultures 58A1 and 58A2 only a small proportion of the flukes were examined, since so little development had occurred, but in the other cultures one quarter of the total number was examined, to ensure that results were directly comparable with those of other cultures.

Table XXXII

Development in serum-free medium, i.e. basic medium (yeast + albumen + G.P.T.).

Culture no.	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Final pH
	Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
58A1	6	0	0	1	0	0	0	0	5	75	5	6.6
58A2	10	0	0	1	1	0	0	0	5	65	5	6.8
59B1	6	1	1	1	0	0	0	0	8	32	6	7.2
59B2	10	2	2	2	0	1	0	0	9	35	7	7.4
71C1	10	1	1	1	0	0	0	0	7	29	5	7.3
71C2	10	1	0	4	0	0	0	0	10	40	6	6.9
71C3	10	0	0	4	0	0	0	0	8	35	7	7.3
TOTAL (out of 70)	62	5	4	14	1	1	0	0				
PERCENTAGE	88	7	6	20	1	1	0	0				

Development in basic medium to which calf serum was added, is shown in Table XXXIII. 8 ml. of 40% calf serum in G.P.T. was included in each culture. The development was again very poor. No sperm occurred in these cultures, although two flukes showed a small amount of vitelline development. Testes, where present, were not large and follicular. Some of the flukes had large, dark-looking degenerating cells around the hold-fast organ.

Table XXXIII

Development in basic medium (yeast + albumen + G.P.T.) + calf serum.

Culture no.	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Final pH
	Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-voir	Eggs				
61A3	1	0	0	0	0	0	0	0	8	60	6	7.6
62A1	4	0	0	1	0	0	0	0	12	65	6	7.3
62A2	8	0	0	2	0	0	0	0	12	65	7	7.5
62A3	10	0	0	8	1	1	1	0	12	100	7	7.2
72C1	4	0	0	0	0	0	0	0	7	30	6	7.1
72C2	10	0	0	7	0	1	0	0	12	65	7	6.8
TOTAL (out of 60)	37	0	0	18	1	2	1	0				
PERCENTAGE	62	0	0	30	2	3	2	0				

Since results have already been given in Table XIV, Section IV, for development in cultures containing horse serum added to basic medium (i.e. 8 ml. of 40% horse serum in G.P.T. + 2 ml. albumen + 1½ ml. of 40% yeast extract), these results are not repeated in detail here. They are, however, included in the summary in Table XXXV. Development in this medium was distinctly better than that in either basic medium with no serum, or basic medium with calf serum.

In order, therefore, to commence investigating which fractions in horse serum are important, horse serum was dialysed, and the result of substituting dialysed horse serum for "whole" horse serum is shown in Table XXXIV. Testes were well developed; active sperm and vitellaria occurred in considerable numbers.

Table XXXIV

Development in basic medium (yeast + albumen + G.P.T.) + dialysed horse serum.

Culture no.	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Final pH
	Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
59A1	10	8	8	9	8	4	3	0	8	30	6	7.5
59A2	10	6	6	10	6	2	1	0	11	45	6	7.5
60A1	10	3	1	9	7	2	0	0	12	70	5	7.0
60A2	10	2	2	9	1	3	0	0	9	40	6	7.0
TOTAL (out of 40)	40	19	17	37	22	11	4	0				
PERCENTAGE	100	48	43	93	55	28	10	0				

A comparison of development in basic medium plus (a) G.P.T., (b) calf serum, (c) horse serum and (d) dialysed horse serum, is given in Table XXXV; summarizing results from Tables XXXII and XXXIII, together with those in Table XIV, Section IV, for basic medium plus horse serum, and those in Table XXXIV, for basic medium plus dialysed horse serum.

Table XXXV

A comparison of development in basic medium to which was added (a) G.P.T. but no serum (b) calf serum (c) horse serum and (d) dialysed horse serum. (Basic medium = yeast + albumen + G.P.T.)

Type of serum preparation	Percentage occurrence of genitalia in selected flukes							
	Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs
(a) None (control)	77	7	6	20	1	1	0	0
(b) Calf serum	62	0	0	30	2	3	2	0
(c) Horse serum	98	35	30	70	38	24	14	0
(d) Dialysed horse serum	100	48	43	93	55	28	10	0

An attempt was made to replace the horse serum with a plasma protein. Table XXXVI compares the effects of substituting (a) G.P.T. and (b) bovine plasma albumin (3% in 8 ml. of G.P.T. per culture) for horse serum.

In all cultures development was poor. The "tails" were fairly long, but very few flukes showed much genital development. The bovine plasma albumin cultures showed no improvement compared with the G.P.T.-containing controls.

Table XXXVI

Development in bovine plasma albumin + basic medium, compared with controls containing basic medium, made up to a similar volume with G.P.T. (Basic medium = yeast + albumen + G.P.T.)

Culture no.	Additive to basic medium	No. of flukes out of 25 taken at random, developing genitalia								Days incubated	Final pH
		Early testes	Follicu-:lar testes	Mature sperm	Active sperm	Uterus	Vitel-:laria	Yolk reser-:voir	Eggs		
85N1	G.P.T.	3	22	0	0	11	0	0	0	6	6.6
85N2	"	2	23	4	3	10	1	1	0	6	6.8
TOTAL		5	45	4	3	21	1	1	0		
PERCENTAGE		10	90	8	6	42	2	2	0		
85B1	bovine plasma albumin	14	11	2	2	3	0	0	0	6	6.7
85B2	"	15	10	0	0	2	0	0	0	6	6.7
TOTAL		29	21	2	2	5	0	0	0		
PERCENTAGE		58	42	4	4	10	0	0	0		

As it had been shown in Tables XXXIV and XXXV that horse serum could be successfully replaced by dialysed horse serum, further fractionation of dialysed horse serum was carried out. Tables XXXVII to XL show the results of a series of experiments using separated horse serum proteins.

Table XXXVII presents results from the control cultures, containing dialysed horse serum, and "blanks" with G.P.T. instead of serum.

Table XXXVIII shows the effect of (a) removing the γ-globulin fraction from the dialysed horse serum, (b) the γ-globulin fraction itself, and (c) dialysed horse serum from which γ-globulins were removed, together with the γ-globulins which had been added once more to the medium. This third set of cultures served as a check on the amount of activity which could be obtained from re-constituted dialysed horse serum after it had gone through the separation processes.

Table XXXIX is similar to Table XXXVIII, but here the α-, β- and muco-globulin fraction was removed, tested by itself, and added once more to re-constitute the dialysed horse serum.

Table XXXVII

Development in dialysed horse serum + basic medium, compared with development in basic medium, made up to a similar volume with G.P.T. These two sets of cultures form controls and "blanks" for experiments using fractionated serum. (Basic medium = yeast + albumen + G.P.T.)

Culture no.	Additive to basic medium	No. of flukes out of 25 taken at random, developing genitalia								Days incubated	Final pH
		Early testes	Follicu-:lar testes	Mature sperm	Active sperm	Uterus	Vitel-:laria	Yolk reser-:voir	Eggs		
84A1		0	25	11	9	21	18	10	0	6	6.6
84A2	dialysed	0	25	18	18	22	14	12	0	6	6.6
84A3	horse	0	25	10	6	23	13	7	0	7	6.4
87 4i	serum	0	25	8	8	19	8	5	0	5	6.7
87 4ii		0	25	14	13	21	12	9	0	6	6.4
TOTAL		0	125	61	54	106	65	43	0		
PERCENTAGE		0	100	49	43	85	52	34	0		
85N1	G.P.T.	3	22	0	0	11	0	0	0	6	6.6
85N2		2	23	4	3	10	1	1	0	6	6.8
88A1		6	19	1	1	9	0	0	0	6	6.5
88A2		17	8	0	0	2	0	0	0	6	6.3
88A3		12	13	1	1	6	0	0	0	6	6.3
TOTAL		40	85	6	5	38	1	1	0		
PERCENTAGE		32	68	5	4	30	1	1	0		

Table XXXVIII

Development in basic medium to which was added (a) dialysed horse serum from which γ-globulin had been removed, (b) the γ-globulin fraction, and (c) re-constituted dialysed horse serum containing both fractions. (Basic medium = yeast + albumen + G.P.T.)

	Culture no.	Additive to basic medium	No of flukes out of 25 taken at random, developing genitalia								Days incubated	Final pH
			Early testes	Follicu-:lar testes	Mature sperm	Active sperm	Uterus	Vitel-:laria	Yolk reser-:voir	Eggs		
a	84B1	dialysed horse serum minus γ-globulin	5	20	3	3	8	2	1	0	6	6.5
	84B2		6	19	1	0	10	3	2	0	7	6.6
	84B3		7	19	1	0	14	2	1	0	7	6.5
TOTAL			18	57	5	3	32	7	4	0		
PERCENTAGE			24	76	7	4	43	9	5	0		
b	84C1	γ-globulin of horse serum	0	25	7	4	24	7	1	0	6	6.4
	84C2		0	25	5	3	23	4	2	0	7	6.3
	84C3		0	25	7	5	23	7	1	0	7	6.5
TOTAL			0	75	19	12	70	18	4	0		
PERCENTAGE			0	100	25	16	93	24	5	0		
c	84D1	dialysed horse serum minus γ-globulin, plus γ-globulin	0	25	8	4	25	10	2	0	6	6.6
	84D2		1	24	5	3	21	4	1	0	7	6.4
TOTAL			1	49	13	7	46	14	3	0		
PERCENTAGE			2	98	26	14	92	28	6	0		

Table XXXIX

Development in basic medium to which was added (a) dialysed horse serum from which α-, β- and muco-globulins had been removed, (b) the α-, β- and muco-globulin fraction, and (c) re-constituted dialysed horse serum containing both fractions. (Basic medium = yeast + albumen + G.P.T.)

	Culture no.	Additive to basic medium	No. of flukes out of 25 taken at random, developing genitalia								Days incubated	Final pH
			Early testes	Follicu-:lar testes	Mature sperm	Active sperm	Uterus	Vitel-:laria	Yolk reser-:voir	Eggs		
	8711	dialysed horse	0	25	13	11	21	3	1	0	5	6.4
a	8712	serum minus α, β,	5	20	4	4	19	2	0	0	6	6.5
	8713	muco-globulin	2	23	9	7	17	1	0	0	6	6.5
TOTAL			7	68	26	22	57	6	1	0		
PERCENTAGE			9	91	35	29	76	8	1	0		
	8721	α-, β- and	11	14	7	3	4	1	0	0	5	6.5
b	8722	muco-globulin	12	13	2	2	7	0	0	0	6	6.4
	8723		15	10	0	0	3	1	0	0	6	6.4
TOTAL			38	37	9	5	14	2	0	0		
PERCENTAGE			51	49	12	7	19	3	0	0		
	8731	dialysed horse	0	25	19	10	23	4	1	0	5	6.5
c	8732	serum minus α, β,	0	25	9	6	25	5	0	0	6	6.5
	8733	muco-globulin plus α-, β-, muco-globulin	0	25	11	6	24	10	4	0	6	6.2
TOTAL			0	75	39	22	72	19	5	0		
PERCENTAGE			0	100	52	29	96	25	7	0		

Table XL compares development in all cultures from the experiments on horse serum protein separations. Only the percentages of flukes showing developmental stages are given.

It can be seen that dialysed horse serum from which γ-globulins were removed (Table XXXVIII) supported but little more development than the "blanks" containing no serum (Table XXXVII). The numbers showing vitelline development were just slightly higher. Cultures in which γ-globulins alone were used, showed a marked improvement, (Table XXXVIII). The numbers producing sperm, and the amounts of sperm produced were much larger, and much larger numbers showed ciliated uteri. Scattered vitelline cells were more common, and the flukes looked more healthy, showing few signs of degeneration. Re-constituted dialysed serum containing γ-globulin showed no improvement over the γ-globulin alone. Sperm and vitelline development was rather poor compared with the dialysed horse serum controls (Table XXXVII).

When the α-, β and muco-globulin fraction was removed from dialysed horse serum, sperm production was still fairly good, although vitelline development was greatly reduced (Table XXXIX). The α-, β- and

Table XL

A summary of results presented in Tables XXXVII, XXXVIII and XXXIX, showing development in various fractions of horse serum, added to basic medium. (Basic medium = yeast + albumen + G.P.T.)

Horse serum (H.S.) fraction	Percentage occurrence of flukes showing genitalia								Estimation of development
	Early testes	Follicular testes	Mature sperm	Active sperm	Uterus	Vitellaria	Yolk reser-:voir	Eggs	
Dialysed H.S. (control)	0	100	49	43	85	52	34	0	good
Dialysed H.S. minus γ-globulin	24	76	7	4	43	9	5	0	poor
Dialysed H.S. minus α-, β- and muco-globulin	9	91	35	29	76	8	1	0	good sperm, poor vitellaria
γ-globulin	0	100	25	16	93	24	5	0	fair
α-, β- and muco-globulin	38	37	9	5	14	2	0	0	very poor
Dialysed H.S. $-\gamma$-globulin $+\gamma$-globulin	2	98	26	14	92	28	6	0	fair
Dialysed H.S. $-\alpha$-, β- and muco-globulin $+\alpha$-, β- and muco-globulin	0	100	52	29	96	25	7	0	fair
No serum in any form ("blank")	32	68	5	4	30	1	1	0	very poor

muco-globulins alone showed little improvement over "blanks" containing no serum (Table XXXVII). The combined fractions, however, gave better results than either of the two fractions separately. The sperm production was comparable with that of the dialysed horse serum controls, although vitelline development was not so great.

Discussion

The addition of horse serum to medium consisting of yeast, albumen and G.P.T., greatly increases development (Tables XXXII, XXXV).

Dialysed horse serum, under the same conditions, completely replaces "whole" horse serum (Table XXXIV, XXXV). This shows that the essential factors in the horse serum are confined to the high molecular weight substances, which consist mainly of the proteins, albumin and globulin. These factors, however, are not necessarily the proteins themselves; it is possible that they are non-protein groups which are bound to the proteins, and may remain associated with them through various fraction-:ation processes. It is clear, however, that none of the dialysable, low molecular weight constituents of the serum are essential in the presence of yeast, albumen

and G.P.T.

It was thought, therefore, that the albumins of serum might be responsible for some of its beneficial effect, but the results in Table XXXVI show that horse serum cannot be successfully replaced by a preparation of purified bovine plasma albumin. This may be an indica-:tion that the albumins of serum are unimportant nutritionally. There is, however, the possibility that bovine albumin might carry a toxic factor. Development in calf serum was very poor, and sperm production was completely lacking (Table XXXIII), suggesting the presence of a toxic substance. The numbers of flukes producing sperm when serum was omitted from the medium, however, were so low that this conclusion is doubtful, and even if true, there is little likelihood of the toxic substance being present in albumin purified from the serum. It seems more likely that bovine serum proteins are unsuitable nutritionally. Robinson (1960) also found that some mammalian sera were suitable as culture media for Schistosoma mansoni, while others were not. Unexpectedly, the greatest numbers of eggs were layed by blood flukes in serum from animals which are poor hosts to S. mansoni.

With regard to the globulin fraction, the experiment in which γ-globulins were separated from the serum (Table XXXVIII) suggests that these were responsible for

much of the activity of dialysed horse serum, and hence for the activity of "whole" horse serum. The serum fraction from which α-, β- and muco-globulins were removed, was more active than the globulin fraction by itself, which promoted very little development (Table XXXIX). This confirms the supposition that activity is mainly confined to the γ-globulins. The α-, β- and muco-globulin fraction also appears to have some activity, since better results were obtained with the re-combined fractions, than with either of the two fractions separately.

In neither of these fractionation experiments was development in the re-combined fractions as advanced as in the control cultures containing dialysed serum. Two possible reasons for this are suggested.

(a) Bacterial contamination which arose during the fractionation procedures (in spite of aseptic precautions) may have altered the serum chemically. Although micro-organisms were destroyed by antibiotics or removed by filtration before the medium was used, bacterial metabolites would have remained in the medium.

(b) The re-dissolved globulins are present in solution as larger particles than in the original serum. They may not be so readily utilized in this form.

The technique used for precipitating the α-, β- and muco-globulin fraction was based on the assumption that the γ-globulins, which precipitated as the ammonium sulphate reached 34% saturation, were re-dissolved when the concentration rose to 50% saturation. The solubilities of the different protein fractions are certainly very sensitive to the concentration of salts present, as was shown when the globulin solutions were dialysed first against water, and then against salt solutions. It is not certain, however, that some of the γ-globulins would not have remained in solution along with the α-, β- and muco-globulins. Before carrying out further culture work using separated proteins, it would be necessary to obtain more precise knowledge of the components of each isolated fraction. It might be an improvement in technique if, as the ammonium sulphate concentration rose, each precipitated fraction were removed, before a further fraction precipitated out.

In spite of these technical difficulties, fractionation of serum proteins may lead to important discoveries about nutritional requirements of helminths.

After precipitation with ammonium sulphate, serum proteins can be further fractionated according to their solubility in water (Cohn et al., 1940). The activity of these fractions could therefore also be tested.

Although ammonium sulphate precipitation techniques provide a good method of preparing fractions in bulk from mixed proteins, many of these fractions are themselves still complex mixtures. Electrophoresis provides a more descriminating method of separating proteins, but it is less easy to obtain large quantities of the purified proteins. By the use of zone electrophoresis, however, it has been possible to separate protein fractions from serum, in sufficient quantities for use in tissue cultures (Katsuta et al., 1959). Zone electrophoresis involves the use of a solid or semi-solid support in the form of a gel or filter paper, through which the charged proteins migrate at different rates according to their electrophoretic mobility. Starch gels have been used

a great deal for the purpose of separating serum proteins (Smithies, 1959). Once separation is complete, the various fractions can be removed by cutting out the appropriate sections of gel, and the proteins extracted from them, either by maceration in a buffer solution, or by first freezing and thawing the gel, which renders it sponge-like, and the proteins can then be centrifuged, squeezed or sucked out.

The most recent refinement which has been used in separating proteins is immunoelectrophoresis (Williams, 1960). Immune serum is allowed to react with the various protein fractions which have been separated electrophoretically in an agar gel. Lines of precipitates are formed, indicating the reaction of each protein with its corresponding antibody in the immune serum. Even the best separations achieved by zone electrophoresis are not so discriminating as those effected by this method, since proteins can be distinguished even although their mobilities are very close, and large concentrations of two or three antigens cannot mask the presence of trace amounts of others. Although this method could not be used for preparing fractions of proteins to be tested as nutrients for organisms in culture, it could be used

as an accurate check on the proteins present in a given fraction. Thus, provided that facilities were available, protein fractions could be separated from horse serum by ammonium sulphate precipitation or by zone electrophoresis, and the constituents of these fractions could be precisely determined by immunoelectrophoresis.

In attempts to arrive at a chemically defined culture medium it is wise to keep a balance between the use of analytical methods for breaking down complex media, and the synthetic approach, i.e. replacing the complex substances with purified products, with a view to discovering suitable substitutes. Unfortunately it is not possible to obtain commercially prepared purified horse serum globulins. Globulin fractions from bovine serum can be obtained, but since the experiments using calf serum showed that it is unlikely that bovine serum proteins are suitable, it seems probable that purified bovine globulin fractions would not provide successful substitutes for horse serum.

Summary

(1) Horse serum, when added to a medium consisting of yeast extract, egg albumen, glucose and balanced saline, has a distinct beneficial effect on development of Diplostomum phoxini.

(2) Calf serum and bovine plasma albumin appear equally unsuccessful in replacing horse serum.

(3) Under the conditions tested, dialysis of the horse serum did not alter its effect.

(4) Some indication is given that the most important elements in the horse serum are associated with the γ-globulins.

(5) The use of zone electrophoresis as a further method of separating serum proteins, for the purpose of isolating the active fraction or fractions, is suggested.

SECTION VII: CULTIVATION OF OTHER SPECIES OF DIPLOSTOMUM

Introduction

Concurrently with work on Diplostomum phoxini, a few experiments were performed on the cultivation of two other species of Diplostomum. The metacercariae of these two species occur, one in the lens, and one in the choroid layer of the eye of the stickleback, Gasterosteus aculeatus. Gray (unpublished) recognised these two metacercariae as distinct species, and identified the lens one as Diplostomum spathaceum (Rudolphi, 1819). The other, he referred to as Diplostomum "c".

Any cultivation technique, however successful, would be of little value in the study of helminth physiology if it could be applied to one species only. If, with minor alterations, the technique may be applied to other helminths, general principles concerning nutritional and physical requirements of helminths may be established. It was felt that the first step in this wider project was to apply some of the techniques established for D. phoxini to other species of Diplostomum. The two species from the stickleback eye

were chosen since a supply of sticklebacks was available.

Culture media tried were:-

Yolk + albumen

yolk + albumen + gelatin

yolk + albumen + casein hydrolysate

autolysed yeast suspension + horse serum + albumen.

The purpose of using gelatin and casein hydrolysate was to discover if any change in the diazo reaction given by the vitellaria occurred, as reported for D. phoxini by Bell (1958). This reaction, as has already been pointed out (page 49) gives an indication, by the production of an intense scarlet colour, of the presence of phenolic egg-shell precursors. Since abnormal yellow-brown diazo reactions are given by D. phoxini matured in vitro, it was of interest to observe the diazo reactions in cultured flukes of another species. Diazo tests on D. phoxini cultured in similar media were also carried out, for comparison.

Materials and Methods

Collection of fish

Sticklebacks were at first obtained from a pond

near Bellshill, Lanarkshire. Unfortunately, in these sticklebacks, although the infection rate was high, the intensity of infection was low, so that only a few experiments could be carried out. Later, a source of more heavily infected sticklebacks was discovered in a reservoir near Lennoxtown, Stirlingshire. These fish were available only in small numbers, and successful collection of large fish was mainly confined to the spring and autumn. The heavier infections which they carried, however, made them more suitable for cultivation purposes.

Procedure

Fish were killed by immersion in iodine-alcohol solution as was done with the minnows. Each fish was pinned out, dorsal side uppermost. The roof of the skull was removed intact with sterile scalpel and forceps; the optic nerves were severed, and the conjunctiva loosened from the surface of each cornea. The eye-balls were then removed to a Petri dish containing balanced salt solution with glucose (G.T. or G.P.T.) where they were cut open, and the vitreous humour allowed to escape. The lenses were removed to separate Petri dishes. When D. spathaceum metacercariae were to be placed in culture, the lenses were teased

open, and immediately transferred to the culture medium. Six lenses were used per culture, giving approximately 25 metacercariae in each culture. When Diplostomum "c" was to be used, the choroid layer was teased into small fragments, and the larvae allowed to emerge. These were collected by pipette and transferred to the culture medium.

Recording results

Results were recorded in a similar manner to that used for yolk + albumen cultures of Diplostomum phoxini, except that the information required was of a more qualitative kind, and more description was used. Counts of dividing cells were used in one case to describe development in very poorly developed flukes. Aceto-orcein squashes, prepared as described by Bell & Hopkins (1956) were employed to show the dividing cells.

Results

Diplostomum spathaceum

Five cultures were set up three with yolk + albumen medium, and two with autolysed yeast + horse serum + albumen medium, as shown in Table XLI.

Table XLI

Culture no.	Medium	Days incubated	No. of flukes recovered alive
3L1	2 ml. albumen 10 ml. yolk containing 20% G.T.	3	11
3L2	"	7	4
3L3	"	12	6
4L1	2 ml. albumen 2 ml. autolysed yeast suspension 8 ml. horse serum diluted to 30% with G.T.	6	4
4L3	"	6	0

Culture No. 3L1

Of the eleven flukes, three had remained in the lenses, and had the appearance of metacercariae. Three others which had emerged from the lenses appeared exactly the same. The remaining five had slightly more pronounced "tails". Aceto-orcein squashes of two of these showed only three cell divisions in one, and two in the other.

Culture No. 3L2

Of the four, two had remained in the lenses, in the metacercarial condition. The gut caeca of the other two were full of yolk globules, and their "tails" appeared twice the length of those which had remained in the lenses. Examination of the living flukes at X 400 magnification showed no genitalia had developed.

Culture No. 3L3

All six appeared slightly larger than metacer-:cariae. One had a longer "tail" than the rest, and its gut caeca were distended with yolk globules. Examination again showed that genitalia had not developed.

Culture No. 4L1

Only one of the four had escaped from the lenses. It showed no development, and was no larger than those in the lenses.

Culture No. 4L3

No living flukes were recovered from this culture.

Tables XLII to XLIV show cultures of Diplostomum phoxini and Diplostomum "c" in various yolk-containing media. Their development in these media is compared.

Table XLII

Cultures of Diplostomum phoxini and Diplostomum "c" in yolk + albumen medium.

Culture	Species	Days incubated	No. of flukes recovered alive
22A	Diplostomum phoxini	6	14
Ca	Diplostomum "c"	6	2

Culture No. 22A

All fourteen gave yellow or brown diazo reactions. A few were slightly reddish-brown.

Culture No. Ca

Both the flukes were surprisingly large, and had long "tail" regions. One had testes with mature sperm, numerous active sperm in the vas deferens, a large ovary, a uterus, extensive vitellaria, and a small yolk reservoir. The other not only showed all these genitalia but had nine transparent, colourless eggs in the uterus. These eggs had pliable "shells" of an appearance similar to cellophane, and under pressure of a coverslip, formed transverse creases as shown in the drawing in figure 7. The outlines of the yolk cells were just visible in some of the eggs.

A yellow diazo reaction was given by both flukes.

Table XLIII

Cultures of Diplostomum phoxini and Diplostomum "c" in yolk + albumen medium containing gelatin. (Yolk con-:tained 20% G.T., in which 2% gelatin was incorporated.)

Culture no.	Species	Days incubated	No. of flukes recovered alive
22C	Diplostomum phoxini	6	9
C gel	Diplostomum "c"	6	11

Culture No. 22C

Eight of the nine gave yellow or brown diazo reactions, but one fluke gave a bright red reaction.

Culture No. C gel

All but one of the flukes had yolk reservoirs, and three had eggs. The eggs were three to five in number, and were abnormally tanned in two of the flukes, but clear in appearance, having a cellophane-like "shell" in the third. The diazo reaction given by the vitellaria in these flukes was mainly brown, but some of them had patches of pink colour.

Table XLIV

A culture of D. phoxini in yolk medium (pH 7.1) containing 1.5% casein hydrolysate in the diluting G.T., and a culture of Diplostomum "c" in yolk + albumen medium, also containing 1.5% casein hydrolysate in the diluting G.T.

Culture no.	Species	Days incubated	No. of flukes recovered alive
46 3a	Diplostomum phoxini	4	16
C cas	Diplostomum "c"	7	11

Culture No. 46 3a

Five were tested for diazo reaction. Of these, two gave an orange-red reaction, one having a particularly bright colour. The others gave dull brown colours.

Culture No. C cas.

Eight of the eleven flukes had eggs. Most of these eggs had dark, abnormal tanning, but a few were more transparent and appeared less abnormal. Active sperm were seen in only one of the flukes.

The diazo reactions given by these flukes were mainly brown, with light red patches.

Figure 7 illustrates differences in egg-shell for-:mation in (a) a normal strigeid egg from a fluke matured in vivo, (b) an egg from Diplostomum "c" in culture, and (c) an egg from D. phoxini in culture. The drawings were made from specimens which had been fixed in 70% alcohol. The dotted areas indicate the shell material, which is contributed by the vitelline cells.

In the formation of a normal egg, a number of vitelline cells from the yolk reservoir surround an ovum, and vitelline globules, which are very obvious while they are still in the yolk reservoir, become no longer visible.

The shell material, which has a pale golden colour, then becomes evenly distributed over the surface of the egg, forming the shell. The walls of the vitelline cells are at first still visible, but these soon disappear, and the egg-shell material starts to "tan". In figure 7, the normal egg is shown with the outlines of the vitelline cells still just visible, and the shell material distri-:buted around the egg, as the even dotting illustrates. Longitudinal creases in the shell, which was still pliable at the time of fixation, are shown.

The egg of D. phoxini cultured in yolk + albumen medium formed no shell. The globules of the vitelline cells remained in situ, as shown by the large dots. Some of the shell material, which was not distributed evenly round the egg, became very darkly tanned, as the solid black shapes indicate. The walls of the vitelline cells did not break down, but remained clearly visible.

The thin, cellophane-like "shell" of the egg of Diplostomum "c" produced in culture, is illustrated by the fine outline, which is greatly creased, showing that this thin "shell" was extremely pliable at the time of fixation. Some of the shell material was seen to be distributed in fairly large patches on the egg surface, as is shown by the dotted areas, and some of the

outlines of the vitelline cells were faintly visible. This egg showed no very dark, abnormal tanning.

These illustrations show that the egg produced by Diplostomum "c" in culture, although far from normal, had fewer abnormalities than an egg produced under similar conditions by Diplostomum phoxini.

FIGURE 7

Drawings of eggs of Diplostomum species made from specimens fixed in 70% alcohol, and mounted in Canada balsam.

Note the appearance of the egg-shells, and of the vitelline cells which were visible in all of these eggs. The ovum was not visible in any of them.

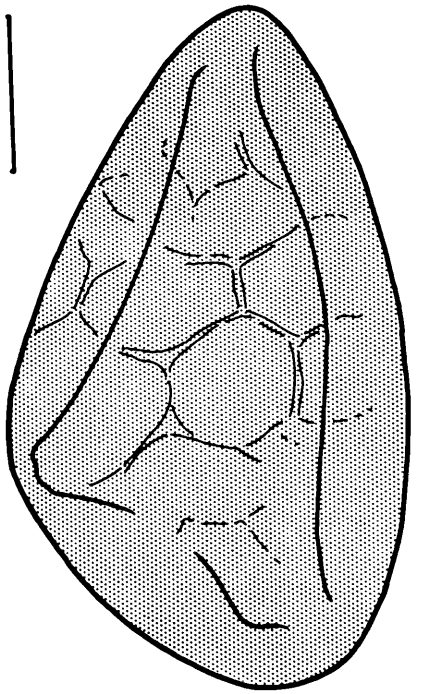
(N.B. see text.)

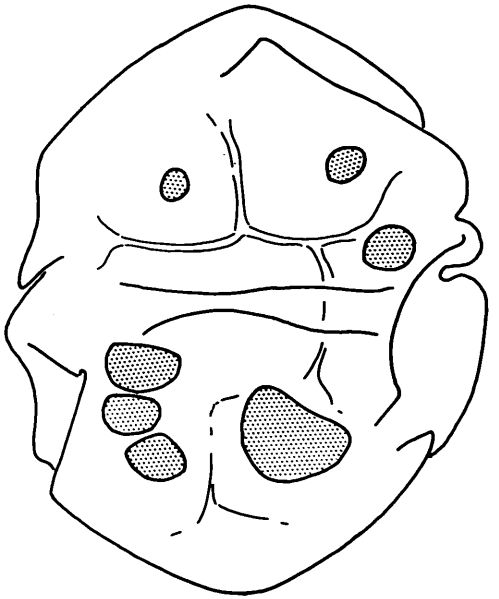
NORMAL STRIGEID EGG (DIPLOSTOMUM PHOXINI)

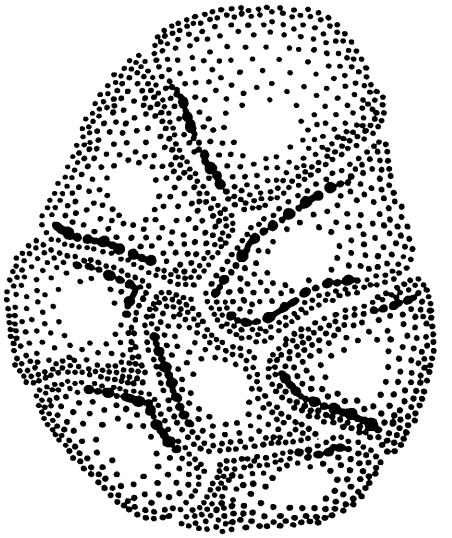
EGG OF DIPLOSTOMUM 'C'. YOLK + ALBUMEN MEDIUM

EGG OF DIPLOSTOMUM PHOXINI. YOLK + ALBUMEN MEDIUM

0.02 MM.







Discussion

Metacercariae occurring in the choroid layer of the stickleback eye, appear to have similar physical and chemical requirements to those of Diplostomum phoxini, since both species develop in the yolk + albumen medium. Metacercariae of Diplostomum spathaceum, however, have different requirements, for although they survive well in this medium, and take substantial amounts of it into their intestinal caeca, no development occurs. This may indicate that the species, D. spathaceum is physiologically more widely separated from Diplostomum "c" than is D. phoxini, although the latter at first sight appears ecologically quite distinct from the other two. However, in comparing the environments of these species, it would be more relevant to compare their habitats in the final hosts, since it is desired to reproduce in culture, development to the adult stage. Hopkins and others* in this laboratory carried out feeding experiments which showed that the herring gull is a suitable host for all three species, but the duck, (Anas boshas domestica) is suitable for D. phoxini and Diplostomum "c" only. Several attempts to infect ducks with the metacercariae from the stickleback lenses were unsuccessful.

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*Personal communication

This again illustrates a difference in the requirements for development to maturity, of D. spathaceum from those of the other species of Diplostomum considered here.

It was confirmed that abnormal yellow and brown diazo reactions are given by vitellaria of Diplostomum phoxini cultured in yolk + albumen medium, as was reported by Bell (1958). Diplostomum "c" gave a similar reaction. With D. phoxini, gelatin and casein improved the colour of the diazo reaction in some flukes, when added to the medium, again confirming Bell's observations. With Diplostomum "c" similar changes in colour reaction occurred. In some cases the colour was bright red, while in others only a light red or pink colour developed. These paler colours suggested only small amounts of the phenolic egg-shell precursors, rather than the presence of abnormal substances. Brown colours, on the other hand, were thought to indicate an abnormal reaction.

An interesting point was that the egg-shell formation in some of the cultured Diplostomum "c" was less abnormal than that observed in D. phoxini, in that the shell material did not remain in clumps in the vitelline cells, and there was little doubt that a very thin

shell was formed. This was in contrast to the abnormal eggs seen in cultures of D. phoxini, in which the vitelline globules remained intact, and tanning of shell material took place in localised areas within the vitelline cells, no real shell being formed. This phenomenom was not correlated with more normal diazo reactions of the vitellaria, although the formation of the trematode egg-shell depends on the oxidation of phenolic substances in the proteins of the shell precursor material, which is supplied by the vitellaria. Little explanation of these observations can be offered until more information is available, but it may be that, owing to some metabolic difference between the two species, the task of producing culture conditions suitable for egg production in Diplostomum "c" will turn out to be an easier one than it is for Diplostomum phoxini.

These results encourage further experiments on other species of strigeids, and should some of the techniques developed become applicable to other groups of trematodes, they may prove useful in a much wider field of research.

Summary

(1) Metacercariae of *Diplostomum spathaceum*, derived from the lenses of sticklebacks, showed a small amount of growth, but no development in yolk + albumen medium. They neither grew nor developed in medium consisting of autolysed yeast, horse serum, egg albumen and balanced saline.

(2) Metacercariae of *Diplostomum* "c" from the choroid layer of the stickleback eye, grew and developed to maturity in yolk + albumen medium. Some of the eggs produced were less abnormal than those produced under similar conditions by *Diplostomum phoxini*.

(3) Diazo reactions given by the vitellaria of *Diplostomum* "c" cultured *in vitro* were found to be similar to those of *D. phoxini*, under similar conditions.

(4) Physiological distinctions between these three species of *Diplostomum* are discussed.

DISCUSSION

The process of maturation of *Diplostomum phoxini* from the metacercaria to the adult is very different from its previous larval development. The latter consists mainly of slow somatic growth, and development of larval structures (e.g. cilia in the miracidium; a tail in the cercaria). In the final host develop-:ment leads to the rapid production of functioning genitalia, accompanied by a small amount of somatic growth. The increase in mass during the first three days in the final host, and the subsequent frequent discharge of eggs and sperm, infer that the flukes must be drawing, at least in part, on an exogenous source of food. This, however, is not the case with every species. The metacercariae of some trematodes form genital primordia, and require little or no nutrient in the final host. This is true of the microphallid trematode, *Gynaecotyla adunca*, which can mature and produce normal eggs in 1% sea water at 40°C (Hunter & Chait, 1952). A medium containing some nutrients was required to stimulate *in vitro* development of sperm and eggs (both abnormal) in *Posthodiplostomum minimum*, although in this fluke most of the genital organs are already present in the

metacercaria in an undeveloped condition (Ferguson, 1940).

In the case of the metacercaria of Diplostomum phoxini, there are no genital primordia. The problem of discovering what conditions stimulate maturation is therefore a more complex one, which is likely to reveal more fundamental information.

When it was discovered that D. phoxini matures to the stage of producing normal active sperm, and abnormal eggs in yolk + albumen medium (Bell & Smyth, 1958), two ways of investigating this problem seemed possible. Attempts might be made to analyse the yolk, and identify the important constituents, or to replace it by simpler substances which could be analysed more easily. Since the use of yolk has many technical disadvantages, the latter approach was preferred.

In the most successful replacement medium, sperm production was better than in yolk or yolk + albumen media, and was often as good as in flukes matured in vivo. However, in only about 5% were eggs formed, and these were abnormal. This medium consisted of balanced saline, glucose, an amino acid solution and three complex natural substances: horse serum, yeast extract and egg albumen. Analytical procedures were

applied to each of these natural substances.

When yeast extract was omitted from the medium, sperm production was greatly depressed, but when serum was omitted, the sperm production was still fairly good in some of the flukes. This indicated that yeast is responsible for the excellent sperm production, but that it cannot be fully utilized if the other constituents are not present. The B vitamins in yeast extract are no doubt important, but dialysis showed that some non-dialysable factor, perhaps a protein, was also important for promoting development.

The γ-globulin fraction in horse serum appears to be important. In the presence of the rest of the medium constituents, the dialysable substances in horse serum are of no importance. This does not necessarily mean that they cannot be utilized, as the same substances may be supplied already by the other constituents of the medium.

Egg albumen is partly, but not completely, replaceable by amino acids. The best results were obtained when albumen and amino acids were added together. This shows that there must be some prosthetic group, or some particular protein chain formation in albumen, which is

used. Also it seems that albumen has a detoxicating effect since fewer dead flukes are found in cultures to which albumen has been added.

It is not likely that all of the twenty amino acids of the synthetic solution used are essential, and these amino acids may be replaced by a more restricted group. However, results of experiments using more restricted ranges of amino acids may be confusing, since apart from the amino acid content of the proteins in the medium, yeast and serum also contain free amino acids.

The yeast + albumen + serum + amino acid medium supplies a useful intermediate step between a yolk and a purely synthetic medium. Some of the essential constituents in the yeast have now been identified, which forms a further step towards a synthetic medium (Williams et al., in preparation).

Two difficulties arose when assessing media. Firstly, the determination of the most suitable criteria to use, and secondly, development is so variable that a large number of flukes must be examined.

The criteria which have been used are based on the presence or absence of certain genitalia. Few

quantitative descriptions of development have been given. If a convenient quantitative method of assessing sperm and vitelline development were used, more precise comparative information about media would be obtained. What can be gained in precision, however, may be paralleled by what is lost in speed of working. For example, it seemed worth while to examine twenty-five flukes taken at random from a culture instead of selecting up to twelve of the most well-developed, but this change in technique reduced the number of cultures which could be examined in one day. It might be profitable not to confine criteria to the genitalia, but to use observations of somatic structures also. In culture the cuticle is often distorted, the holdfast organ laden with granules, and the excretory system distended with fluid. Useful comparisons might be made by basing criteria on such observations.

It is probable that the variability in the developmental stages reached by the flukes is, in many cases, largely due to the use of fresh egg albumen in the culture medium, Albumen varies in consistency from one egg to another, and is by no means homogeneous within one egg. It is therefore impossible to administer a completely equivalent portion of albumen

to each culture. The most liquid albumen dissolves in the culture medium, but denser fractions remain as undissolved masses. Albumen is strongly alkaline (pH about 9.0) and so its presence renders difficult the control of the pH of the whole medium. Although efforts were made to make the medium more homogeneous, such as Seitz-filtration of the albumen, it is probable that conditions will not be standardized until a synthetic substitute is found for the egg albumen. However, even when albumen is left out of the medium, leaving it totally homogeneous, a few flukes produce active sperm and yolk reservoirs; a number scarcely develop beyond the metacercarial condition, and the rest show varying intermediate stages of development. This suggests a variation in the intrinsic capability of the metacercariae to develop. On the other hand, in yolk + albumen medium, which contains a richer supply of most of the essential nutrients, much less variation in development occurs, the majority of flukes reaching the stage of vitelline development. Thus, in very poor media, uniformly poor development occurs, in good media, uniformly good development takes place, but in media which are satisfactory for some requirements, but poor in other respects, development varies widely. In an ideal medium,

therefore, the majority of flukes would show normal development, and intrinsic differences between the flukes would not be so obvious.

Some of the work described in this thesis is no more than a preliminary trial of experiments which could well be repeated with improved technique. An example of this is the use of protein fractions separated from serum. The result of this experiment was sufficiently interesting to make it seem a worth while project to repeat and verify the work already done, and to devise new experiments using different fractions of serum proteins. Further experiments on the culti-:vation of other species of trematodes would also be interesting, since only a small number of cultures were devoted to this, and only species closely related to Diplostomum phoxini were used.

A number of other projects could have been carried further, but it seemed wiser at this stage to carry out a fairly wide range of experiments, probing several different aspects of the problem, rather than to attempt quantitative determinations of requirements for any single substance. For instance, it seemed pointless to continue attempts to discover which amino acids were essential, while it was still necessary to

add to the medium, complex naturally occurring substances which contain a variety of amino acids and proteins.

The medium consisting of yeast, serum, albumen and G.P.T., contains proteins contributed by the albumen and serum, carbohydrate in the form of glucose, and some lipids from the serum. The yeast contains water-soluble vitamins. Although there may be some important hormones present in fresh serum, these would probably be destroyed after storage of the serum. It would therefore be of interest to study the effects of adding hormones to the medium. Although little or no work has been reported on the effect of hormones on helminths grown in culture, interesting observations have been made on the effects of some of these substances on cultured cells. Vogelaar & Erlichman (1933), in an attempt to replace embryo extract as a source of growth-promoting substances in cell culture media, produced a feeding solution for thyroid fibroblasts, which contained insulin and thyroxin, along with hemin, cystine, peptone and irradiated beef plasma. Following this lead, Baker (1936) produced what he termed an "artificial" medium for fibroblasts, epithelial cells and monocytes, which also contained insulin and thyroxin. Von Haam and Cappel (1940a, b), using Tyrode's solution as a basic

medium, tested out the effects of various hormones on cultured fibroblasts. They found that while estrin, progesterone, testosterone and thyroxin had very little effect on growth, and adrenalin and cortin were inhibitory, insulin brought about a very marked increase in the rate of growth. It was pointed out by Morgan (1950) that the use of hormones in cell culture has not persisted in more recent synthetic media. Franks, however, at the inaugural meeting of the British Tissue Culture Association, 1960, stated that the observations on the growth-promoting effect of insulin have been confirmed by recent workers. These observations give sufficient indication that it would be worth while to attempt to apply some of this work, in particular the use of insulin, to helminth cultivation.

It was mentioned in the Introduction (page 7) that as a result of the work of Aldrich et al. (1954) it appeared probable that the tissues of tapeworms, as well as the tissues of their hosts are subject to hormonal effects. Other instances of parasites being influenced by the hormones of the host are known. Encystation of Opalina in the frog rectum was shown by McConnachie (1960) to be closely correlated with shedding of sperm by the frogs, and it was suggested that the

frogs' gonadal hormones were responsible for bringing about encystation. The trematode, Polystoma, lays its eggs only when the host frogs are spawning. Miretski (1951) showed that egg-laying in Polystoma was stimulated even when spawning in the host was induced out of season by injection of frog pituitaries, again suggesting that the parasite is directly influenced by the host's hormones.

In vitro cultivation should provide a good method for studying such effects.

SUMMARY

(1) The advantages of using *in vitro* cultivation as a method for investigating helminth physiology are described.

(2) *Diplostomum phoxini* metacercariae were removed from minnow brains and cultured aseptically in various media.

(3) The optimum pH range was determined for two different media. A pH around 7.0 was found to be most suitable for flukes developing in a hen egg yolk + albumen medium, whereas a pH in the region of 6.4 was more suitable when the flukes were cultured in a medium consisting of yeast extract, horse serum, egg albumen and balanced saline. The explanation of these different pH optima is discussed.

(4) Roller tube culture vessels were compared with universal containers, and found to be less suitable.

(5) Mouse liver and pigeon mucosa extracts failed to improve development, but autolysed yeast, when added to albumen and horse serum, promoted considerable development.

(6) Each constituent of the yeast + albumen + horse serum medium was treated in turn to determine (a) its precise effect on development, (b) whether any procedure such as heating, dialysing or fractionating affected its activity, and (c) whether it could be replaced by a purified chemical substance or substances. Autolysed yeast was replaced by a heat-stable extract of yeast, and dialysis of this yeast extract did not altogether destroy its activity. Albumen was partially replaceable by an amino acid solution, but there is evidence that it has a detoxicating effect in the medium. Preparations of casein, gelatin and plasma albumin were ineffective in replacing egg albumen. Horse serum promoted development, and dialysed horse serum was equally effective under the conditions used. Some evidence is presented showing that the active constituents of horse serum are associated with the γ-globulin fraction.

(7) The part played by each constituent of the medium, in the nutrition of the flukes, is discussed.

(8) In a medium consisting of yeast extract, egg albumen, horse serum, amino acids and glucose-saline, amounts of sperm produced exceeded those in yolk + albumen, and often equalled those <u>in vivo</u>.

The few eggs produced, however, were abnormal in that no shell formation took place.

(9) Metacercariae of two other species of Diplostomum were placed in culture. In one case (D. spathaceum) no development occurred, while in the other (Diplostomum "c") active sperm and eggs were produced within five days. Several of the eggs produced formed thin shells. Diazo staining reactions were observed in cultured Diplostomum "c", and compared with results previously reported by Bell & Smyth (1958) for diazo reactions in D. phoxini in vivo and in vitro. They proved to be similar.

(10) Some plans for future work on the cultiva-:tion of helminths are suggested.

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