STUDIES ON THE NUTRITIONAL REQUIREMENTS OF *DIPLOSTOMUM PHOXINI* (STRIGEIDA TREMATODA) *IN VITRO*

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

A.A.	Amino acids.
Alb.	Egg albumen.
ALE.	Autoclaved liver extract.
BEL.	Butanol extract of liver.
BEELE.	Butanol extracted, ethanolic liver extract.
BSS.	Balanced salt solution.
BSSG.	Balanced salt solution with glucose added.
B vit.	B vitamins.
EE_{50}.	50% embryo extract.
EEL.	Ethanol extract of liver.
HS.	Horse serum.
P.A.B.A.	Para amino-benzoic acid.
RLE.	Raw liver extract.
YE.	Yeast extract.
Basal media	(a) In Sections V & VI; BSSG + egg albumen.
	(b) In Sections I, II & IV; BSSG + egg albumen + horse serum.
	(c) In Section III; BSSG + egg albumen + horse serum + amino acids.

INTRODUCTION

The physiology of intestinal helminths has long interested parasitologists because of the economic importance of this group of animals. One aspect which has received considerable attention has been the nutritional requirements of parasitic worms; but in spite of much work, comparatively little is, as yet known. Intestinal worms of mammals and birds inhabit an environment characterised by a low oxygen tension, a constant temperature, usually except close to the mucosa, a variable pH (Read, 1950). Many live in close association with other worms and all are exposed to secretory fluids of the host's intestine. In short the environment presents very variable conditions in contrast to the tissue habitat, and may vary as much if not more than the environment inhabited by free-living animals.

A number of workers attempted the elucidation of the growth requirement of intestinal helminths <u>in vivo</u> by studying the effects on the growth rate of adding or withholding certain compounds from the host's diet. A more recent refinement of this method has been the use of substances labelled with radioactive isotopes and their subsequent measurement in the parasite. Of the various compounds tested, vitamins have received most

attention. In studies of the vitamin requirement of the fowl nematode, Ascaridia galli, Sadun et al. (1949) found pronounced growth retardation of worms in chicken fed on a purified diet containing minimal amounts of vitamin B_{12}. Supplementing the diet with liver extract led to a better, though subnormal growth. As liver has a high B_{12} content, they assumed that the nematodes require vitamin B_{12} or a substance present in liver for normal growth.

Beaver (quoted by Von Brand, 1952) found that development in the trematode, Echinostum revoltum, was greatly retarded in pigeons kept on a diet deficient in vitamins A and D, a clear indication that the diet of the host directly influences the parasite.

The nutritional requirements of the rat cestode, Hymenolepis diminuta have been investigated by Hager (1941), Chandler (1943), Addis & Chandler (1944, 1946). These workers fed cysticercoids to rats and after the worms had attained maturity, the effect of the diet of the host on proglottid formation observed. Elimination of proteins from the host's diet was without effect on the parasites; but omission of carbohydrates had a profound effect. Of various vitamin deficiences tested, only lack of B vitamins affected growth adversely. The adverse

effects were only observed in female rats and castrated males (Addis & Chandler, 1946). It was concluded that carbohydrates and some vitamins were the important compounds absorbed by the tapeworm from the diet of the host, as distinct from nutrients obtained from the mucosal secretions.

The above observations demonstrate physiological relationships between parasite and host, however, they do not necessarily show true nutritional requirement. For example the slower rate of growth when vitamin B_{12} was absent from the diet (Sadun et al., 1949) could indicate a nutritional requirement by the parasite for this vitamin, but the correlation could be more indirect. A low B_{12} intake might well affect the bacterial flora and so bring a change in the chemical and physical environment of the worm. Similarly, the experiments, in which Addis & Chandler (1946) found absence of B vitamins caused stunting of the worms in castrated but not normal male rats, indicate that it is dangerous to assume a direct correlation between the experimental procedure and the effect observed. In the case of the latter experiments it would seem likely that normally, enough B vitamins reach the worms in the intestinal lumen through the mucosa. In castrated animals, however, the amount

reaching the lumen from the tissue falls below the amount required by the worm, so that the worm becomes dependent on the B vitamins in the diet. There are of course many other possible ways of interpreting the observations. Addis (1946) reported that the administration of testosterone or progesterone to castrated rats resulted in normal sized worm regardless of whether the hosts were kept in a normal or a deficient diet and concluded that gonadotropic hormones were important.

Since it is almost impossible to standardise the various processes which occur within the host, the interpretation of the results of in vivo experiments on nutritional requirements of helminths is extremely difficult.

Some workers have used the study of the chemical composition as a basis for the determination of nutritional requirements. One of the most significant findings in this approach has been the discovery that the pyridoxine content of some intestinal helminths was higher than that which occurred in the liver of their hosts. Chance & Dirnhuber (1949) carried out quantitative microbiological assays of some B vitamins in the trematode, Fasiola hepatica, the cestode, Moniezia benedeni, and the nematode, Nippostrongylus muris. They found that the

worms contained thiamin, nicotinic acid, pantothenic acid and riboflavin in amounts smaller than occurred in the liver of their respective hosts, but found more pyridoxine in the parasites than in the host liver (Table 2). They therefore assumed that pyridoxine was an important substance in the metabolism of intestinal helminths and suggested that a correlation probably existed between the demands for this compound and the high rate of egg production, since pyridoxine is involved in the processes leading to protein synthesis.

Nyberg (1952) carried out quantitative microbiological assay of vitamin B_{12} in Diphyllobothrium latum and Taenia saginata and found that the former contained as much as fifty times the B_{12} content of the latter. This probably demonstrates that B_{12} was more important in the metabolism of D. latum than that of T. saginata. By feeding radioactive vitamin B_{12} to patients infected with D. latum and T. saginata respectively, Nyberg (1958), was able to show uptake of B_{12} was mainly concentrated in the proximal part of D. latum but, this was not so in T. saginata. In view of the fact that absorption of the vitamin occurs chiefly in the proximal part of the intestine, Nyberg assumed that the worms located in the duodenum or in the proximal part of the jejunum are in a much better position, from the standpoint of the supply of B_{12} than worms in

distal sites.

There are, however, several limitations in this approach. Three obvious ones may be mentioned.

(a) It is impossible to gain any idea of the rate of metabolism by determining the amount of any compound present at one time.

(b) Certain compounds present in appreciable quantities might be excretory rather than nutritive products.

(c) Labile substances may be lost or transformed during chemical analysis.

Most of the difficulties encountered in the two methods previously described could be eliminated if the worms are cultivated in vitro, though only in bacteriological sterile media could nutritional requirements be determined with some degree of certainty. This approach has been widely used to study growth requirements of parasitic protozoa and recently defined media has been developed for the maintenance of two species belonging to the Trypanosomidae (Von Brand, 1956). Smyth (1946 et seq.) and Hopkins (1952) demonstrated and discussed the value of in vitro cultivation for investigating the ways in which the gut environment controls the maturation

and development of cestodes and trematodes.

Little progress has so far been achieved in attempts to maintain adult intestinal helminths *in vitro*. On the other hand growth has been achieved *in vitro* starting with larval stages. Smyth (1949, 1950, 1954) succeeded in developing plerocercoids of *Schistocephalus solidus* and *Ligula intestinalis* to sexual maturity *in vitro*. In these two species of tapeworms nearly all growth and development occurs in the plerocercoid stage. Not surprisingly therefore, Smyth found that the endogenous store of nutritive material was sufficient to permit development to maturity as soon as the physico-chemical environment was favourable. Since maturation could occur in a non-nutritive medium little knowledge of the nutritional requirement of these worms was obtained. The same is true of the microphallid trematode, *Gynaecotyla adunca*, which was reared to sexual maturity in 1% sea water at 40°C (Hunter & Chait, 1952). However, in the culture of parasitic nematodes and strigeid trematodes, it has been found that the nutritive components of the medium are as important as the provision of a suitable physico-chemical environment.

Stoll (1953) successfully cultivated the nematode, *Neoaplectana glaseri*, in a fluid medium containing veal or

beef heart infusion broth supplemented with raw acid liver extract. Weinstein & Jones (1956), Silverman (1959) using almost identical media consisting of chick embryo extract, liver extract, casein hydrolysate and serum were able to culture *in vitro* the histotrophic stages of parasitic nematodes. The latter worker used the sheep nematodes, *Haemonchus contortus* and *Ostertagia* spp., and the former workers used the rat nematode, *Nippostrongylus muris*.

Fergusson (1940) was the first to culture successfully *in vitro* the metacercariae of strigeid trematodes to sexual maturity, although the sperms and eggs were found to be abnormal. Using the metacercariae of *Posthodiplostomum minimum*, adult trematodes were obtained in a few days at 39°C in a medium consisting of dilute tyrode solution, chicken serum and yeast extract. *Diplostomum phoxini* developed to maturity in a medium consisting of yolk and albumen of hen egg (Bell & Smyth, 1958). The eggs produced by the flukes were, like those produced by *Posthodiplostomum*, abnormal. Later Wilson (1960) found that the yolk in this medium could be replaced partly by yeast extract and horse serum.

The media used for all the cultivation work just described were of biological origin, and much too complex

to allow the identification definite growth factors. Smyth (1959) pointed out that the stage so far reached in the *in vitro* cultivation of helminths corresponded to that attained in the early days of tissue culture when responses to particular media under defined physical conditions were being established. Before factors promoting growth and development can be discovered and accurately defined, it is essential that the media used should be composed of substances which are themselves defined rather than those of complex material derived from natural sources.

The aim of the work described in this thesis was to elucidate some of the nutritional requirements of the strigeid trematode, *Diplostomum phoxini*, developing to maturity. This problem was approached by replacing the components of successful media of biological origin, by chemical defined substances. The advantage of using larval strigeid trematodes for *in vitro* cultivation has already been discussed by Bell & Hopkins (1956), and also criteria for assessing development in the trematode, *Diplostomum phoxini*, have been established by Bell & Smyth (1958).

Although hen egg yolk and albumen medium (Bell & Smyth, 1958) supported good development of the flukes, there are several disadvantages in its use. Technically it is difficult to recover flukes from cultures containing

yolk globules. Furthermore, as yolk is an extremely complex substance its fractionation, to determine the components which are essential for the growth of the flukes, is extremely difficult. Two substances, yeast extract and horse serum which could be more easily analysed were substituted for the yolk by Wilson (1960). She obtained her best results in a medium consisting of glucose balanced salt solution, amino acids, yeast extract, egg albumen and horse serum. Sperm production of the flukes in this medium was better than in hen egg yolk and albumen medium and just as good as in flukes matured in vivo. However, egg production in the yeast medium was considerably lower than in the yolk and albumen medium. The replacement of the three natural products in Wilson's medium, namely egg albumen, yeast extract and horse serum, by defined substances forms the main theme of this thesis.

The thesis is divided into eight sections; the first four being devoted to replacement of yeast in the medium and the improvement of the level of development in a yeast medium. Sections five and six are devoted respectively to the replacement of horse serum by other natural products and the study of horse serum as a nutrient. The probable role of albumen in the medium is discussed in section seven. The last section reports an investigation of flukes which had begun the final phase of development

in vivo and were then placed in culture. It was decided to discover whether these flukes reached maturity any more successfully than metacercariae taken from the minnow's brain. Finally results of the various sections are summarised and discussed.

The Biology of Diplostomum phoxini (Faust, 1918)

The life cycle was first reported by Arvy & Buttner (1954) who also described the morphology of the adult worm (figure 1). A more detailed description of the adult morphology was later given by Rees (1955). The natural definitive host in this country is not yet known; but adult worms have been obtained experimentally in ducklings, (Rees, 1955) herring gulls, mice (Berrie, 1960) and pigeons (Turnbull, unpublished). Significant differences are known to occur in size of flukes and production of eggs in different hosts (Berrie, 1960).

The adult worms reside in the anterior portion of the small intestine of the final host lying partially embedded in the mucosa. Eggs pass out in the faeces. Development and hatching occurs in water probably in the presence of oxygen. The amount of oxygen required for hatching is not known. It takes 12 days for metacercariae to hatch out from eggs incubated at 26°C (Bell, 1958). No details of the description of the miracidium has been

published and details of its development in the molluscan host is lacking.

The molluscan host in Britain is Lymnaea pereger whilst Lymnaea auricularia is the first intermediate host in France. Furcocercous cercariae are known to be developed from sporocysts which form a tangled mass extending throughout the digestive gland and the gonad of the snail host. The sporocysts are elongated, unbranched and white in colour (Rees, 1957). The percentage infection of snails found infected near Aberystwyth (Wales) was approximately four, Arvy & Buttner obtained a similar result recording an infection of 4-5% in L. auricularia in a region near Paris where all the minnows were parasitised.

On emergence from the snail host the cercariae penetrate the tissues of the second intermediate host, the minnow (Phoxinus phoxinus). Rees (1957) suggested that the cercariae are transported from the point of penetration to the brain via the blood stream. The cercariae develop into metacercariae of the diplostomulum type in 28 days (Arvy & Buttner, 1954). Another strigeid cercaria was found to take a much longer time (40-90 days) to develop into the metacercaria diplostomulum (Erasmus, 1958). It

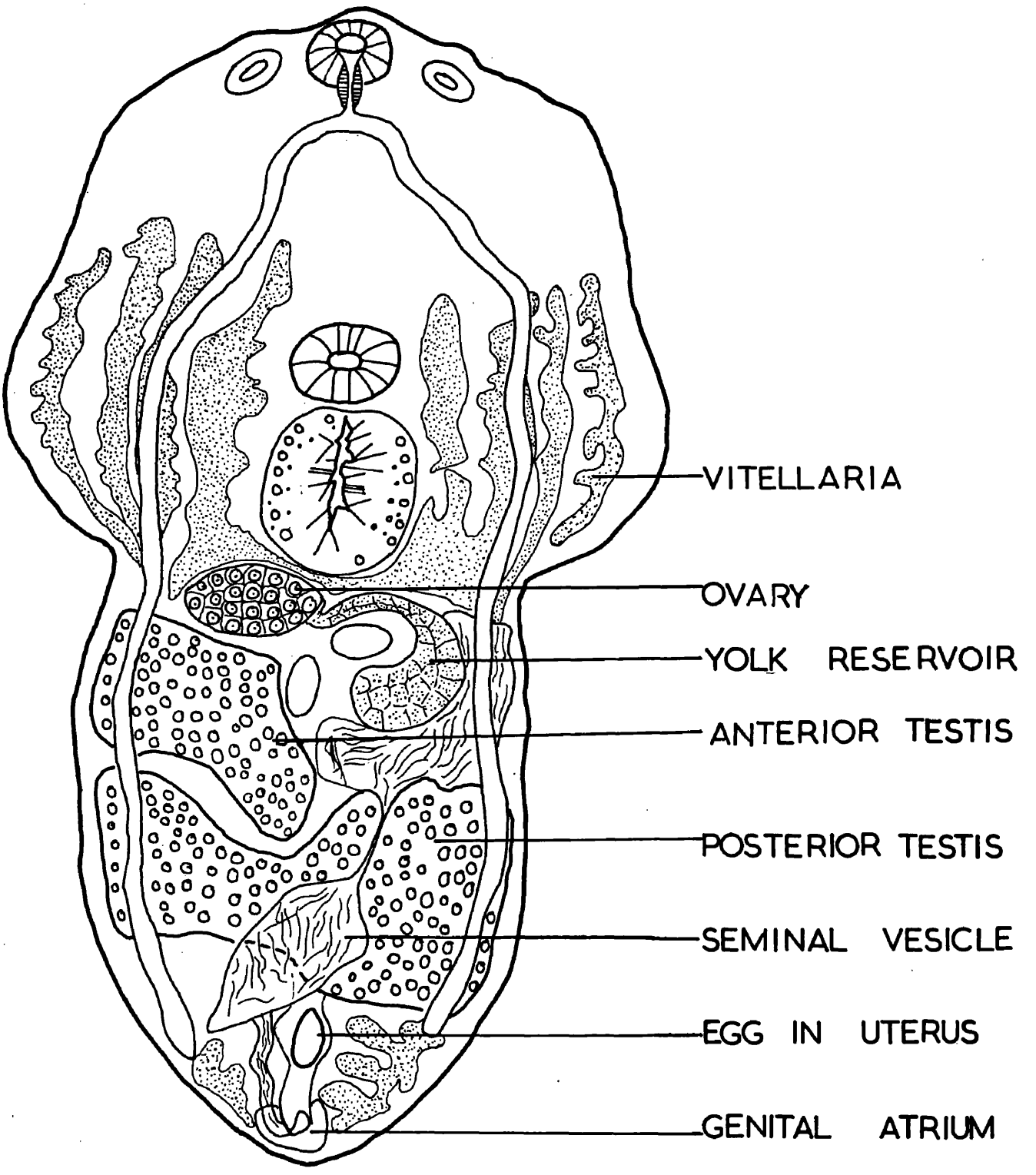
is not known how long it takes the metacercaria to become infective. A precise knowledge of the time and temperature within which the metacercaria becomes infective would be extremely useful for cultivation studies; as this would make it possible to provide infective metacercariae for cultures. In old and heavy infections, metacercariae accumulate in the third and fourth ventricles, optic lobes, lobi inferiores, corpora striata, under the epithelium covering certain areas of the brain and in the proximal regions of the spinal cord (Rees, 1957). Metacercariae of D. phoxini are very common in the Glasgow area of Scotland with an incidence of infection which is almost 100%. It occurs in the brain of minnows found in Loch Lomond, Loch Eck, River Clyde, River Endrick, and the Craigton Burn at Milngavie (Berrie, 1960).

A description of the metacercaria under the name of Tetracotyle phoxini was first given by Ashworth & Bannerman (1927). A more detailed description was later given by Rees (1955). The functions of the lateral organs on either sides of the oral suckers and the adhesive organ which first appears in the metacercariae but persists in the adult is still obscure. Very recently, Lee (unpublished) has demonstrated by means of histochemical test that the cells around these organs secrete a none specific esterase. Since some esterases are known to have proteolytic

properties it may also be interpreted that these organs secrete proteolytic enzymes. Some flukes exude enzymes into the host tissues causing these to degenerate and dissolve into a nutritive fluid which is then ingested by the flukes (Hyman, 1951). Szidat (1929) suggested that this method was characteristic of strigeids which grasp the host's intestinal villi between the adhesive organ and body wall causing congestion and the rupture of the blood vessels of the villi. The exuding blood is then ingested by the fluke and later the villi dissolve furnishing a nutritive pabulum for the parasite.

Metacercariae develop into adult worms when infected minnows are eaten by fish-eating birds. Bell & Hopkins (1956) have described the adult development of D. phoxini in a laboratory host, the duck. On ingestion by a warm-blooded host further development is initiated presumably, by a sudden rise of temperature. Development in the definitive host is characterised by an outburst of intense mitotic activity culminating in the formation of the genitalia. Early genital primodia can be seen by the end of 24 hours, and about 40-48 hours, after entering the definitive host, spermatozoa are visible. The presence of egg shell precursor in vitelline cells can be detected histochemically after 55-60 hours and oviposition occurs between 60-72 hours after infection.

The size of adult flukes recovered from mice, duck and herring gulls has been measured by Berrie (1960). In herring gulls in which he obtained his largest adult flukes with most eggs in the uterus, the mean length of the adult worm was about 1.93 mm.


VITELLARIA
OVARY
YOLK RESERVOIR
ANTERIOR TESTIS
POSTERIOR TESTIS
SEMINAL VESICLE
EGG IN UTERUS
GENITAL ATRIUM

GENERAL MATERIALS AND METHODS

Cultivation Glassware Equipment, Maintenance and Sterilisation

144 1 oz screw-topped universal containers.

144 Pasteur pipettes.

20 20 ml. pipettes.

20 10 ml. pipettes.

10 5 ml. pipettes.

20 crystallising dishes (3½").

20 large petri dishes (3").

20 small petri dishes (2").

12 graduated centrifuge tubes (5 ml.).

50 125 ml. screw-topped bottles.

2 sintered glass filters.

10 150 ml. conical flasks.

10 250 ml. conical flasks.

5 1 ml. syringes.

5 5 ml. syringes.

5 20 ml. syringes.

All new glassware except special ones such as syringes, were immersed for a couple of days in concentrated sulphuric acid, rinsed to remove all traces of acid before subjecting to routine cleaning.

Routine Cleaning

(1) Glassware was boiled in a stainless steel bucket

containing calgon and sodium metasilicate solution for 20-30 minutes. A stock solution (X100) calgon + sodium metasilicate was prepared by dissolving in 5 litres of tap water:

360 g sodium metasilicate

40 g calgon

250 cc of stock was diluted to 25 litres with tap water.

(2) After boiling the calgon was partially decanted and the glassware washed under running water for several hours.

(3) The glassware was transferred into a stainless steel rack which was immersed in an acid bath containing 0.2 N HCl (figure 2) for at least one hour.

(4) Flasks and 125 ml. bottles removed from the rack were washed by hand whilst the rack containing other glassware was transferred to the automatic washer (figure 2) in which washing was carried out for about four hours.

(5) Pipettes were dried by electric pipette drier (figure 2); other articles were dried in an oven at 100°C.

Rubber liners from screw-capped containers and metal

caps were cleaned by boiling.

Sintered glass filters were cleaned by heating to 90°C in concentrated sulphuric acid with 0.5% $NaNO_3$ and 0.5% $NaClO_3$ added. After heating for 20 minutes the filters were left overnight in the solution and then washed by filtering several litres of tap water followed by a litre of distilled water.

Sterilisation of Glassware

(a) Crystallising dishes were covered with 4" petri dish covers.

(b) All pipettes were plugged with non-absorbent cotton wool. The ends of all large pipettes were wrapped; Pasteur pipettes were placed direct in glass cylinders.

(c) Petri dishes were wrapped and flasks and centrifuging tubes plugged with cotton wool and wrapped.

(d) The caps of screw-capped containers were slackly screwed in places.

(e) Sintered glass filters were wrapped.

All of these were sterilised by autoclaving at 110°C for 15 minutes.

Syringes, needles and dissecting instruments were

sterilised in boiling water.

Setting up of Cultures

All aseptic work was carried out where possible in a sterile cabinet fitted with an ultra-violet lamp (figure 3). The strigeid metacercariae were obtained from the brains of minnows (Phoxinus phoxinus) caught in the River Clyde, east of Hamilton. The fish were kept in aerated running water aquaria, and fed with dried Daphnia and minced liver. The minnows were maintained in this condition for periods up to about two months.

Minnow brains were removed aseptically by the method described by Bell & Hopkins (1956) teased out in balanced salt solution to liberate the metacercariae, which were pipetted into media in culture tubes. Between 50-140 flukes were usually used per culture. Cultivation was carried out in one ounce screw-topped containers held in a water bath (figures 4a & 4b) at 39.5 $\pm$ 1°C. The cultures were shaken intermittently at about 150 r.p.m. for 10 seconds in every minute. The volume of medium usually used per culture was 11-14 ml. The majority of experiments ran for 6 days but some cultures were examined on the fifth or seventh day. The medium was not changed.

Methods of Examining Cultures

In early experiments, cultures were assessed by selecting 8-12 flukes which appeared best developed when viewed under x16 magnification. The flukes were then examined under x200 magnification for genitalia. In order to compare different media the results were recorded as the proportion out of 10, which developed the various genital structures. The structures looked for were testes, mature and active sperms, ovary, uterus, vitellaria and yolk reservoir. The ovary was the most difficult structure to locate in fresh preparations and hence lower values are usually recorded for this than other female genitalia.

There were several limitations inherent in this method of assessment. These have already been discussed by Wilson (1960). One obvious one being the fact that it does not differentiate between a culture containing say only ten flukes with fully formed genitalia and one in which about 30-40 had developed similar structures.

In later experiments the value of a medium was assessed by determining the degree to which the genitalia had developed in a sample. Samples were obtained by recovering 20-25 flukes at random, or as nearly so as possible. Dead flukes were excluded from the sample.

The following is an example of how the cultures were assessed. Of 25 flukes examined in a good culture, 25 might develop follicular testes, i.e. testes with distinct follicles. 19 active sperms, 16 vitellaria and 15 yolk reservoirs. This would be recorded as 100% follicular testes, 76% active sperms, 64% vitellaria and 60% yolk reservoir. This method was more satisfactory than the former one. Almost all media previously assessed by the first method were re-assessed by random sampling.

Only bacteriologically sterile cultures were examined. Bacterial contamination was detected by a significant lowering of the pH, by the appearance of cloudiness in the medium, or by the presence of bacteria in a sample of medium examined under oil immersion lens. Bacterial contamination was confirmed by the examination of a smear of the medium stained with methylene blue.

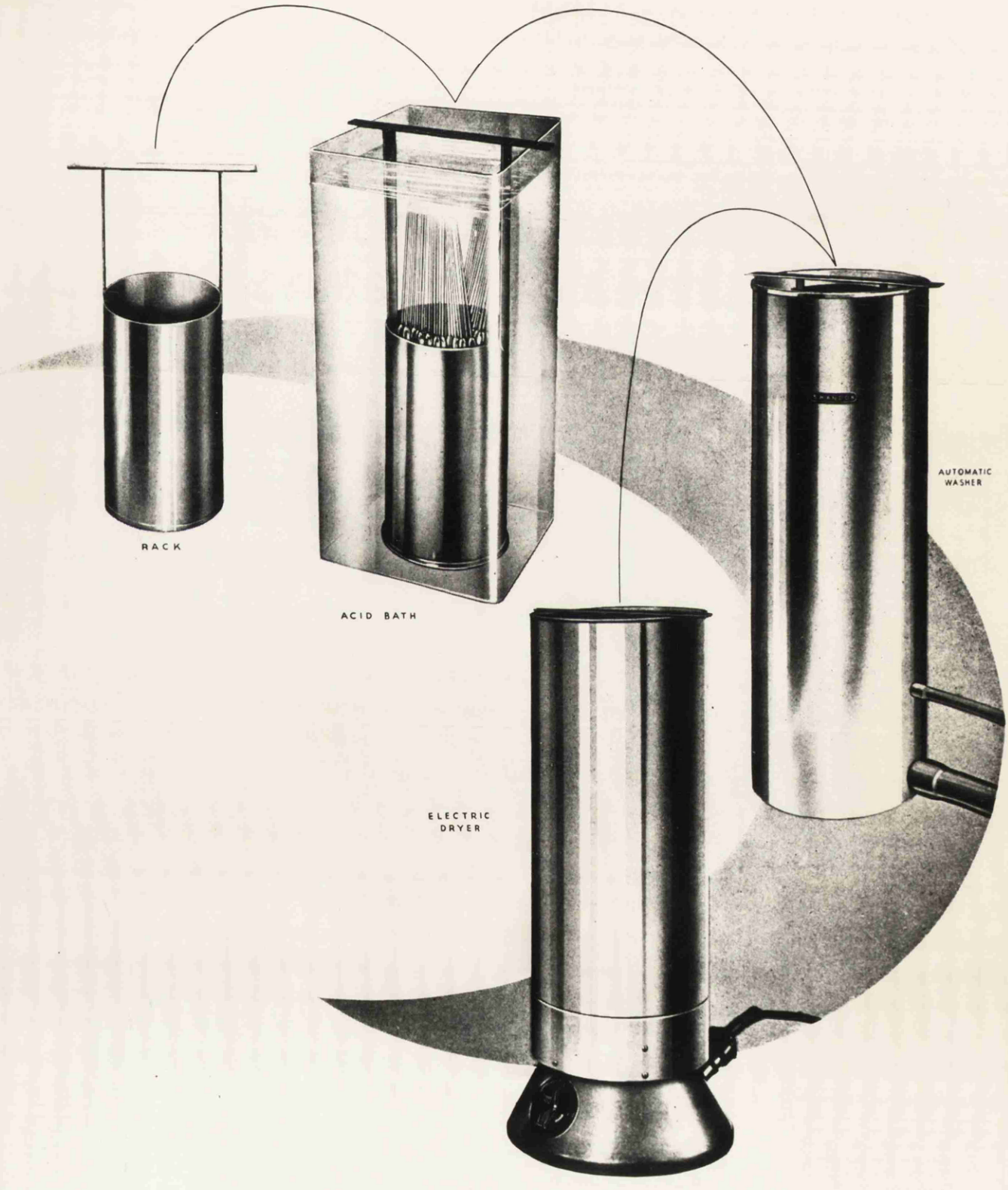
Preparation of Media

Glucose balanced salt solution (BSSG)

A modification of Tyrode solution was used. Extra glucose was added, the bicarbonate omitted and phosphate increased. Two different methods of preparation were used depending on the method of sterilisation.

FIGURE 2

Equipment for pipette washing and drying supplied by The Shandon Scientific Company, London. This equipment was also used for the washing of glassware other than pipettes, as described in the text.


RACK
ACID BATH
AUTOMATIC WASHER
ELECTRIC DRYER

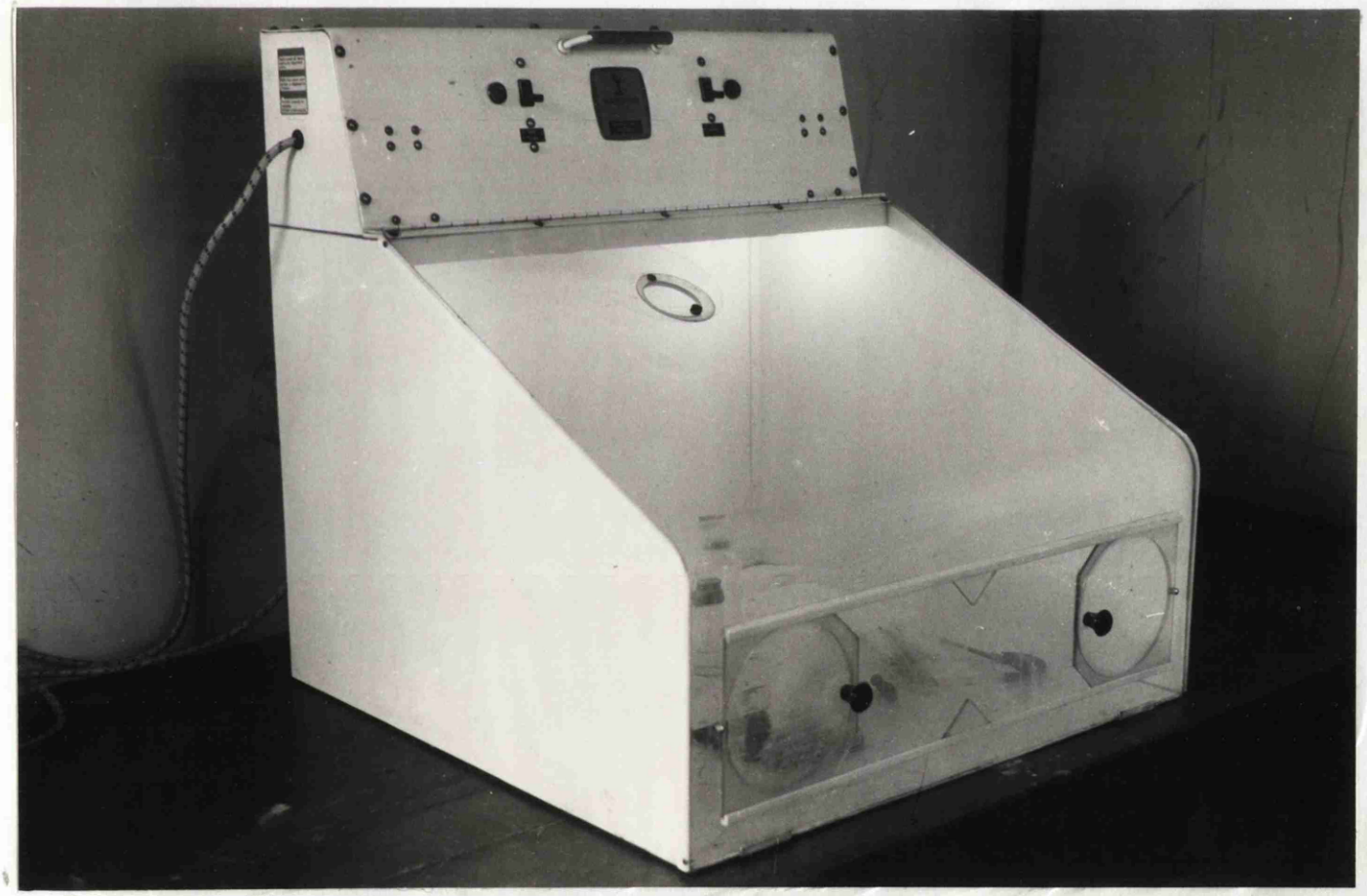


FIGURE 3

Sterile cabinet used for setting up cultures. The cabinet is fitter with an ultra-violet lamp.

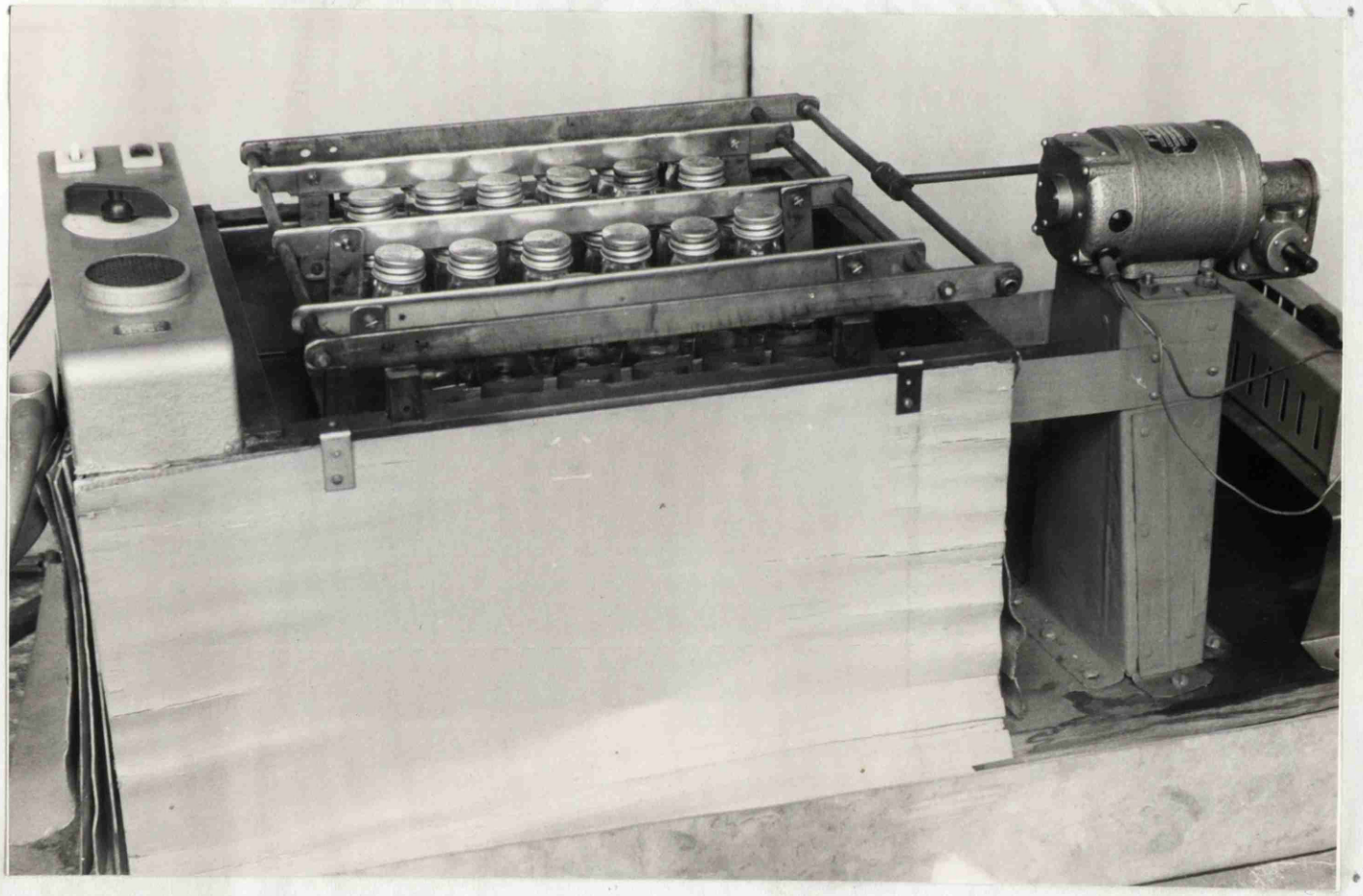


FIGURE 4a

Water bath with a shaker device (in situ) used for D. phoxini cultures.

Capacity:- 36 1-ounce screw-top vials (universal containers).

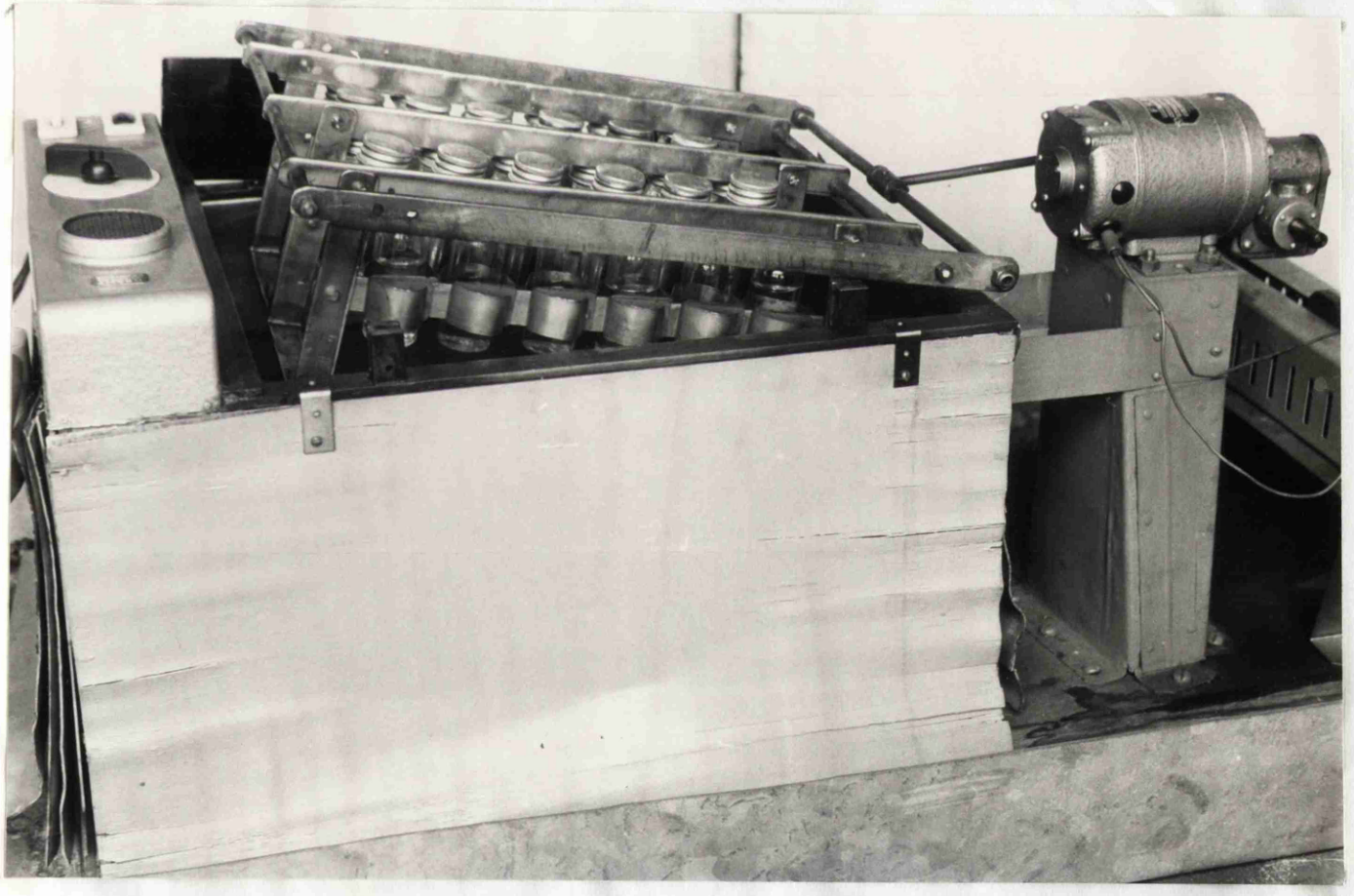


FIGURE 4b

Water bath with shaker displaced to show universal containers in clips.

Method 1

(a) the following salts were dissolved in 960 ml. distilled water.

Sodium chloride (NaCl)	7.26 gm.
Potassium chloride (KCl)	0.20 gm.
Magnesium chloride ($MgCl_2.6H_2O$)	0.10 gm.
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	0.18 gm.
Sodium phosphate (Na_2HPO_4)	0.30 gm.
Calcium chloride ($CaCl_2.6H_2O$)	0.40 gm.

(b) ten grams of glucose were dissolved in 40 ml. distilled water.

The two solutions were added together and the mixture was sterilised by seitz filtration under positive pressure and dispensed in 100 ml. aliquots.

Method 2

The solution was prepared in three parts.

Solution 1

The following salts were dissolved in 900 ml. distilled water.

Sodium chloride	8.8 gm.
Potassium chloride	0.2 gm.
Magnesium chloride	0.1 gm.

Sodium dihydrogen phosphate	0.2 gm.
Sodium phosphate	0.06 gm.

Seventy-two milligrams of phenol red (using a x100 stock) were added and the solution dispensed in 36 ml. aliquots and autoclaved at 110°C for 10 minutes.

Solution 2

Calcium chloride $CaCl_2.6H_2O$ (0.3 gm.) was dissolved in 105 ml. distilled water, dispensed in 4 ml. aliquots and autoclaved at 110°C for 10 minutes.

Solution 3

Twelve grams of glucose were dissolved in 200 ml. distilled water, dispensed in 10 ml. portions and sterilised at 110°C for 10 minutes.

To obtain 40 ml. of BSS, one part of solution 1 was added to solution 2.

Since 6% glucose is approximately isotonic to BSS, glucose solution could be added without affecting the osmotic pressure. Glucose was added to give a level of 1% in the medium.

Albumen

Albumen was removed aseptically from a fresh hen egg

by a method similar to that described by Bell (1958). The shell was painted with alcoholic iodine and dried. A half-inch disc of shell was removed from the end of an egg with sterile scissors. The albumen was either poured out from the shell or removed by pipette into a crystallising dish. Only the liquid fraction was used which can be sucked up with a fine Pasteur pipette. 1-1.5 ml. albumen was added to each culture.

Yeast

Yeast extract was prepared about twelve times final concentration by dissolving 400 mg. of dehydrated water soluble portion of autolysed fresh yeast (Difco) in 10 ml. water and sterilised for 15 minutes at 15 lbs per sq.in. 1 ml. was added to each culture of approximately 12 ml. to achieve a final concentration of about 3.3 mg. per ml.

Horse serum

Number two serum supplied by Burroughs Welcome prepared from re-calcified plasma was obtained in a sterile form and stored up to two months at 4°C. Serum was normally used at a concentration of 25-30% in media.

Amino acid

The amino acid supplement used was basically that recommended by Parker in his synthetic medium 703

(Healy et al., 1954). The stock solution was prepared in two parts.

Amino acid solution 1

This was prepared 10 X final concentration by dissolving the following amino acids obtained from B.D.H. in 100 ml. distilled water.

	mg.
l-arginine monohydrochloride	70
l-histidine monohydrochloride	20
l-lysine	70
dl-tryptophane	20
dl-phenylalanine	50
dl-methionine	30
dl-serine	50
dl-threonine	60
dl-leucine	120
dl-isoleucine	40
dl-valine	50
dl-glutamic acid monohydrochloride	150
dl-aspartic acid	60
dl-alpha-alanine	50
l-proline	40
l-hydroxy proline	10
glycine	50
sodium acetate ($CH_3COONa.3H_2O$)	94

The solution was heated to 80°C in a water bath to dissolve the amino acids, then cooled to room temperature and the following dissolved successively with stirring.

	mg.
l-glutamine	100
cysteine hydrochloride	260
l-ascorbic acid	50
glutathione	10

The solution was sterilised by filtration through sintered glass and dispensed in 10 ml. portions in universal containers.

Amino acid solution 2

This was prepared 20 X final concentration.

	mg.
l-tyrosine	40
l-cystine	20

were dissolved in 100 ml. of 0.075 N HCl, heated to about 65°C filtered through sintered glass and dispensed in 10 ml. portions. 1 ml. of solution 1 together with 0.5 ml. of solution 2 was added to 10 ml. of medium to give approximately the concentrations of tissue culture medium 703.

Antibiotics

Although aseptic precautions were observed whilst setting up cultures, it was found that the addition of antibiotics as a routine procedure to media reduced considerably the chances of bacteria developing in cultures, accidently infected. The following antibiotics were dissolved separately in 100 ml. distilled water, dispensed in 4 ml. aliquots and stored in a frozen state at -15°C.

100,000 units sodium benzylpenicillin (Glaxo)
1 gm. (1,000,000 units) streptomycin sulphate (Glaxo)

The solutions were made up 100 X and 200 X respectively their final concentrations in media. Thus there was a final concentration of 10 units penicillin and 50 units streptomycin per ml. medium. These concentrations of antibiotics appeared to have no effects on the development of the flukes.

pH indicator

Phenol red - 0.2% 100 X stock solution

A stock solution was prepared as described by the Tissue Culture Staff (1955).

0.4 gm. phenol red (B.D.H.) was dissolved in 22 ml. N/20 NaOH. The final volume was increased to 200 ml.

with distilled water and the pH adjusted to 7.0. This 100 X solution was incorporated in BSS as described above and in other solutions to be sterilised. Autoclaved aliquots of 2 ml. were held as stock, and 0.1 ml. was added for each 10 ml. of medium.

The routine incorporation of phenol red in media provided a simple but not very accurate check on the hydrogen ion concentration.

pH

All pH measurements were made by means of a glass electrode. This work was commenced shortly after Wilson (1960) had established that the flukes developed best in yolk-albumen medium having a final pH of 7.0-7.3. Therefore in early experiments the initial pH of culture media was adjusted to this range by the addition of a few drops of 0.2N HCl or 0.2N NaOH. The final pH of cultures was measured about 15-20 minutes after it had been removed from the water bath. However, the medium was not kept in air-tight containers during this period and it was observed that there was a gradual rise in the pH. The method was therefore abandoned and in later work the pH was measured almost immediately the culture tube was opened. By measuring the pH immediately after

the cultures were removed from the water bath, the author found that good development could be obtained between the pH range 6.0-6.9 in a medium consisting of horse serum, yeast extract, amino acid and glucose balanced salt solution. As the pH dropped considerably during the period of cultivation it was not possible to determine a precise optimum pH.

As a routine practice in setting up cultures in later experiments, all culture media were adjusted to a pH of 6.9 before the flukes were added. The final pH of these cultures were often found in the pH range 6.2-6.7.

SECTION I: THE REPLACEMENT OF YEAST EXTRACT IN CULTURE MEDIA BY A SYNTHETIC B VITAMINS SOLUTION

Introduction

The components of media which have been used for the *in vitro* cultivation of *Diplostomum phoxini*, albumen and yolk (Bell & Smyth, 1958) albumen, yeast and serum (Wyllie *et al.*, 1960) are complex, unknown and variable in composition and hence it has been virtually impossible to reproduce similar conditions in cultures. In order to overcome this variation and to determine the nutritional requirements of the flukes, known chemical compounds have to be substituted for complex biological substances in media. The most difficult unknown factors with which to cope are the labile complexes which are either broken down during attempts at chemical separation or by heat during sterilisation. This appears to be the case with albumen. A variety of procedures have been applied (e.g. solidification by heat (Hopkins, unpublished), dialysis and precipitation of some of its proteins (Wilson, 1960)), in attempts to simplify and standardise the albumen supplements, but a decrease in variability has always been associated with a decrease in the general level to which development proceeds.

However, the growth factors in yeast extract have been found to be water soluble and thermostable and hence their replacement by chemically defined substances was considered to be an easier problem than the replacement of the albumen or serum. There were two ways of investigating this problem: (i) by analysing the yeast in an attempt to identify its important constituents, and (ii) by an empirical approach, replacing yeast by substances known to be present in it which have been shown to be essential to various animals and micro-organisms in vitro. The latter approach was tried first and the results are described in this section.

Yeast contains a large number of growth factors, such as amino acids, B vitamins, carbohydrates and peptones (Sykes, 1956). However as most of these compounds occur in significant amounts in the basal medium (glucose, amino acid supplement, albumen and serum) it was felt that the nutritive value of yeast extract might mainly be due to its high B vitamin content. This idea was based on the fact that several workers have shown that B vitamins would replace yeast in various media for the in vitro cultivation of certain parasitic protozoa. While considerable work has been carried out to determine the B vitamin requirements of parasitic protozoa in vitro, little has been done with parasitic worms. However, Weinstein & Jones (1956)

who succeeded in rearing Nippostrongylus muris to adults in axenic conditions, showed that some water soluble vitamins are essential for growth.

This empirical approach to study whether a solution of B vitamins could give the same activity as yeast extract when added to the basal medium was designed therefore to determine whether water soluble B vitamins, additional to those in the basal medium, were required by D. phoxini. When a solution of B vitamins was found to improve development, attempts were made to isolate the active vitamins by a method of elimination. As some B vitamins occur in significant amounts in albumen (Table 3), it was considered that more of these at least were probably not required.

Materials and Methods

B vitamin solutions

All the B vitamins used unless otherwise stated were obtained from B.D.H.

The original solutions used (B vitamin solutions I and Ia) were modifications of Waymouth's solution for Tissue Culture Medium MB 752/1 (quoted by Paul, 1959). Subsequently when it became necessary to evaluate smaller

groups and individual vitamins, eight different vitamin solutions were made (solutions II-VII).

B vitamin Solution I

The following vitamins were successfully dissolved in 100 ml. distilled water by stirring with a glass rod at room temperature to form a concentrated stock solution.

	mg.
Thiamin hydrochloride	40
Calcium pantothenate	4
Riboflavin	4
Pyridoxine hydrochloride	4
Folic acid	1.6
Biotin	0.1
m-inositol	3.7
Nicotinamide	4
Vitamin B_{12}	0.8

As some of the riboflavin, biotin and folic acid did not dissolve the solution was filtered, using a Whatman's No.1 filter paper, before sterilising by passing through a sintered glass filter. 10 ml. portions were collected and stored at -15°C in the dark for periods up to six weeks. In early experiments 0.25 ml. of this stock solution was added per 10 ml. of culture medium

(similar concentration to that used by Waymouth), in later experiments 0.5 ml. was added per 10 ml. of culture medium (i.e. stock solution 20 x concentrated).

B vitamin Solution Ia (Used only in experiment 91)

(1) 0.6 mg. of crystalline biotin (N.B. Ohio) was dissolved in 100 ml. distilled water at 50°C.

(2) Cooled to 40°C and riboflavin (1.0 mg.), nicotinamide (20.0 mg.) were dissolved.

(3) Cooled to room temperature and the following dissolved by successively stirring.

	mg.
Calcium pantothenate	20.0
Pyridoxine hydrochloride	20.0
m-inositol	20.0
B_{12}	3.3
Folic acid	8.0
Thiamin hydrochloride	200.0

(4) Solution was sterilised by passing through a sintered glass filter collected in 10 ml. portions and stored at -15°C. 0.2 ml. of this stock solution was used per 10 ml. of culture medium (i.e. stock solution 50 x concentrated).

Supplement to B vitamin Solution I

The following vitamins not present in solution 1 were dissolved in 100 ml. distilled water and sterilised by filtration through sintered glass:

2.5 mg. para amino benzoic acid

25.0 mg. choline chloride.

0.5 ml. of stock solution was used per 10 ml. of culture medium (i.e. stock solution 20 x concentrated).

Vitamin stock solutions II-VII were prepared as follows, all at 40 x final concentration.

B vitamin Solution II

Pyridoxine hydrochloride (20 mg.) folic acid (0.8 mg.) B_{12} (0.4 mg.) calcium pantothenate (2.0 mg.) thiamin hydrochloride (20.0 mg.) were dissolved in distilled water (25 ml.).

B vitamin Solution III

This was solution II without B_{12} and thiamin hydrochloride.

B vitamin Solution IV

This was solution II without B_{12}, thiamin hydrochloride and calcium pantothenate.

B vitamin Solution V

Pyridoxine hydrochloride (20 mg.) dissolved in distilled water (10 ml.).

B vitamin Solution VIa

Pyridoxal hydrochloride (obtained from Light's) (20 mg.) dissolved in distilled water (10 ml.).

B vitamin Solution VIb

As solution VIa but using pyridoxal hydrochloride from N.B. Ohio.

B vitamin Solution VIc

Pyridoxal hydrochloride (Light's (20 mg.) dissolved in distilled water (250 ml.).

B vitamin Solution VII

Pyridoxamine dihydrochloride (20 mg.) (Light's) dissolved in 10 ml. distilled water.

Solutions II-IV were sterilised by filteration through sintered glass and solutions V-VII were sterilised by autoclaving at 110°C for 15 minutes.

Media

1. Basal medium

The basal medium used in this section consisted of

albumen, horse serum and glucose balanced salt solution (BSSG). To 10 ml. of horse serum in BSSG (serum added to give a concentration of 30%) 1.5 ml. of albumen were added. In earlier experiments the pH was adjusted to 7.2 but in later ones to 6.9.

2. Basal + amino acid medium

1.5 ml. of amino acid were added to 11.5 ml. basal medium and the pH corrected.

3. Basal + amino acid + B vitamin media

To the medium in (2) the appropriate quantity of the required B vitamin solution was added.

4. Basal + yeast medium

1.0 ml. of a 4% autoclaved yeast extract (see general methods) was added to the basal medium.

5. Basal + amino acid + yeast

To medium (4), 1.5 ml. amino acid solution was added.

Table 1

B vitamins content of yeast extract*

Vitamin	Autolysed brewers yeast dry & salt free (Sykes) µg/g	Yeast extract "Difco" µg/g
Thiamin	260	3.2
Riboflavin	80	19.0
Niacin	-	279.0
Nicotinamide	65	-
Panthothenic acid	120	-
Pyridoxine	60	20.0
Folic acid	28	-
Biotin	8	1.4
Choline	10,000	

*Analysis is incomplete

Table 2

Vitamins in parasitic worms. (After Chance & Dirnhuber, 1949)

Parasite	Content µg/g dry weight whole worm				
	Thiamin	Nicotinic acid	Pantothenic acid	Pyridoxine	Riboflavin
Nippostrongylus muris	20.0	119 (383)	19.0 (238)	MB C 13.0 15.5 (10.0)	84 (41)
Ascaris lumbricoides	- 10.0	220 168 (600)	11.2 51.2 (55)	20.0 17.5 - - (13)	14.0 13.0 (50)
Fasciola hepatica	16.0	293 (430)	9.0 (91)	29.0 33.5 (14)	3.25 (52)
Monezia benedeni	8.6	19.0	10.0	7.1	no assay

Figures for vitamin contents of livers of host animals are in parenthesis.

MB = microbiological assay

C = chemical estimate

Table 3

B vitamin content of albumen compared with the contents of B vitamin solution I.

Vitamin	Approximate quantity in 1.5 ml. albumen* of hen's egg - µg	Quantity of vitamin added to 10 ml. final medium - µg +
Thiamin	absent (a)	200
Riboflavin	7.5	20
Nicotinic acid	7.5	20
Panthothenic acid	1.04	20 (Ca^{++} salt)
Pyridoxine	absent (b)	20
Biotin	0.08	0.5
Folic acid	-	8

(a).....Romanoff & Romanoff, 1949 p. 623.

(b).....Romanoff & Romanoff, 1949 p. 629.

*These values were calculated on the assumption that the density of albumen = 1.0. The density of albumen of fresh chicken's egg is 1.035 (Romanoff & Romanoff, 1949).

+Half this concentration of vitamins was used in early experiments.

Results

Table 4 compares the level of development when B vitamin solution I and yeast were added separately to the basal + amino acid medium. It also shows the degree of development attained in the basal + amino acid medium alone.

The general level of development attained in the basal + A.A. medium was the production of testis. Less than 20% of the best 10 flukes developed a uterus as well. On addition of the B vitamins to this medium, the level of development was raised; about 6% developed active sperms, 80% a uterus and 33% vitellaria; but development was not as good as when yeast was added, in which 100% of the flukes had a uterus and 90% vitellaria. This suggests that the B vitamins were partially replacing the activity of yeast in the medium. It will be seen that very low values were recorded for sperm production in experiment 4, but this was probably due to the method of mounting the flukes for examination. Later it was found that a much wetter preparation together with the use of a brighter light, made it easier to observe whether a testis had started producing sperms. Using female genitalia, i.e. uterus, vitellaria and yolk reservoir, for assessing development in media, it was observed that there was a greater variation - (inter-culture variation)

in the basal + A.A. + B vitamins medium than in the yeast medium.

Only cultures 4 V 1, 7 V 1 and 12 V 2 out of the ten cultures in which a vitamin solution was used to replace yeast gave a fairly good development, i.e. only about one culture in every experiment.

The causes for such variation was next investigated.

Table 4

A comparison of the level of development attained in basal + AA + yeast extract with that obtained in basal + AA + B vitamins

Culture no.	Additive to basal + amino acid 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	Vitel-:laria	Yolk reser-:voir	Eggs				
7A1	none	10	-	-	3	-	2	-	-	9	20	6	6.9
7A2		10	-	-	1	-	-	-	-	9	23	7	6.8
7A3		10	-	-	1	-	-	-	-	9	25	7	7.3
	PERCENTAGE	100	0	0	17	0	7	0	0				
4V1	B vitamin solution I 0.025 ml./ml.	10	3	-	10	9	9	8	-	9	31	6	6.7
4V2		10	-	-	8	-	-	-	-	6	14	6	6.8
4V3		10	-	-	10	-	-	-	-	6	12	7	7.5
7V1		10	3	2	9	8	6	6	-	12	35	6	7.2
7V2		10	4	2	8	8	2	1	-	12	46	7	7.2
7V3		10	2	-	9	7	5	3	-	8	24	6	7.6
7V4		10	-	-	10	2	2	-	-	10	42	7	6.9
12V1		10	1	-	3	1	2	1	-	9	43	6	6.8
12V2		10	6	2	9	6	6	6	-	10	60	5	7.1
12V3		10	1	-	4	1	1	1	-	9	30	5	6.9
	PERCENTAGE	100	20	6	80	42	33	26	0				
4X1	yeast	10	-	-	10	10	10	10	-	7	20	7	7.4
4X2		10	-	-	10	10	6	6	-	7	17	6	6.8
4X3		10	6	-	10	9	9	9	-	9	46	6	6.7

As it was felt that the inter-culture variation in the vitamin supplemented medium might be due to inadequate amounts of vitamins, development was studied in basal + A.A. medium supplemented with twice the concentration of B vitamins. The final concentration of B vitamins now being 0.05 ml. stock solution/ml. medium. As controls, a medium consisting of basal + yeast extract was used. The effects of adding choline and amino benzoic acid to the B vitamins medium was also studied - results are shown in Table 5. Since the amino acids in yeast extract added to a culture of 12.5 ml. amounted to approximately 27 mg. (Sykes, 1956, p.17) and that supplied by the amino acid supplement to the medium was only 16 mg. it was decided in comparing yeast and B vitamins media, that the amino acid supplement should be left out of the yeast medium.

The results (Table 5) show that development in basal + A.A. + B vitamins was as good as in basal + yeast medium. Such good results obtained in the B vitamins medium was attributed to the increased concentration of vitamins used in the experiment, which also tended to reduce variation in development of flukes in cultures containing identical medium.

Choline and para amino benzoic acid added as a

supplement to B vitamin solution I did not seem to have any beneficial effect on the level of development of the flukes.

Table 6 shows the development when B vitamins solution I was added to the basal + amino acid medium to give a final concentration of 0.025 ml. and 0.05 ml. stock solution/ml. medium respectively.

Table 5

Shows the effect of adding yeast extract (controls), amino acids + B vitamin solution I and amino acids + B vitamin solution I + para-amino-benzoic acid (PABA) + choline to basal medium (BSSG + HS + alb).

Culture no.	Additive to basal	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				
14E1	yeast extract	10	6	6	10	7	7	6	-	10	43	5	7.1
14E2		10	6	6	10	8	8	6	-	12	60	6	7.2
14E3		10	7	7	10	7	10	6	-	8	42	6	7.1
	PERCENTAGE	100	63	63	100	73	83	60	0				
14C1	A.A. + B vit.Sol.I 0.05 ml./ml.	10	6	6	10	7	9	4	-	8	40	6	7.2
14C2		10	10	5	10	9	10	9	1	10	56	6	7.3
14C3		10	8	5	10	10	10	10	1	10	60	5	7.2
	PERCENTAGE	100	80	53	100	87	97	77	7				
14A1	A.A. + B vit.Sol.I + PABA + choline	10	5	5	10	5	6	3	-	8	40	5	6.6
14A2		10	6	6	10	5	6	4	-	8	36	6	7.2
14A3		10	5	5	10	5	6	3	-	8	39	6	7.3
	PERCENTAGE	100	53	53	100	50	60	33	0				

Table 6

Shows the effect on development of *D. phoxini* when low and high concentration of B vitamins are added to the basal medium + amino acids.

Experiment no.	Total no. of cultures	Conc. of B vit.	Percentage occurrence of genitalia in selected flukes						
			Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitellaria	Yolk reservoir
47, 13 (Table 4) 8 (Table 40)	13	0.025 ml./ ml.	100	28	8	83	26	38	29
14 (Table 5 22 (Table 7)	5	0.05 ml. / ml.	100	70	50	100	72	82	62

Having overcome some variations in cultures which appeared to be due to inadequate supply of vitamins, it was considered worthwhile at this stage to elucidate the important B vitamins in solution I as it was felt that the activity of the solution was due to one or more individual vitamins. This was done by eliminating individual vitamins from solution I gradually and testing the activity of the remainder.

Thiamin, folic acid and pyridoxine are three B vitamins absent from albumen (Romanoff & Romanoff, 1949), and it therefore seemed that these in particular might be important. In addition pantothenic acid has been reported in variable amounts and no value for B_{12} has been found. It was decided therefore to retain calcium pantothenate and B_{12} at the concentration already used. The concentration of thiamin in B vitamin solution I was high (20 µg/ml. of medium), while that of folic acid was high as could easily be achieved (because of its low solubility). The concentration of pyridoxine, however, was relatively low and it was decided to increase this. Four different B vitamin solutions using various combinations of these five vitamins were prepared , viz. solutions II, III, IV and V, as described in the methods. The activity of each when added to the basal

+ amino acid medium was tested. Results are shown in Table 7.

There appeared to be no significant difference among the various media tested. (A difference of less than 20% in the degree of development was considered insignificant when cultures were assessed on the best 10 flukes.) Some variation was observed between cultures containing the same medium although not of the same order of magnitude as those obtained in earlier experiments with the vitamin medium. For example, development in the basal + B vitamin solution I medium was slightly poorer than that obtained in Table 5. This variation may have been due to other constituents of the medium, e.g. albumen or serum.

The most important feature in Table 7 is that solution V containing only pyridoxine gave just as good a result as solution I which contained nine other vitamins including pyridoxine. This suggested that pyridoxine was the most active vitamin in solution I. The concentration of pyridoxine in the medium supplemented by solution V was 50 µg/ml. medium whilst that in the media containing solutions II, III and IV respectively was 20 µg/ml. and that containing solution I was only 2 µg/ml. This shows that a high concentration of pyridoxine had no adverse effects in the development of the flukes.

Table 7

Development in basal + amino acid medium supplemented with various B vitamin soluti

Culture no.	Additive to basal + A.A.	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. i culture
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs		
22V1	B vit. sol. I	10	5	3	10	4	4	4	-	8	38
22V1		10	6	6	10	6	8	4	-	5	25
	PERCENTAGE	100	55	45	100	50	60	40	0		
22R1	B vit. sol. II	10	10	10	10	6	10	9	-	10	56
22R2		10	6	4	10	5	6	6	-	8	36
28C1		10	10	10	10	10	10	10	-	10	55
28C2		10	10	10	10	10	10	10	-	10	53
	PERCENTAGE	100	90	85	100	78	90	88	0		
28P1	B vit. sol. III	10	10	10	10	9	10	10	-	10	45
28P2		10	10	10	10	10	10	8	-	10	52
30E1		10	6	6	9	3	-	-	-	8	25
30E2		10	7	7	10	7	6	6	-	10	66
	PERCENTAGE	100	83	83	98	73	65	60	0		
30F1	B vit. sol. IV	10	5	5	10	3	3	3	-	10	42
30F2		10	6	5	10	1	2	-	-	8	28
90P1		10	10	5	10	10	10	10	-	10	90
90P2		10	10	6	10	10	10	10	-	10	120
	PERCENTAGE	100	78	53	100	80	63	58	0		
30P1	B vit. sol. V	10	5	5	9	3	3	2	-	10	38
30P2		10	10	10	10	6	7	5	-	10	47
35X1		10	8	8	10	10	10	10	-	10	48
35X2		10	6	5	10	7	6	6	-	10	50
	PERCENTAGE	100	73	70	98	65	65	58	0		

B vitamin solution I = original solution
B vitamin solution II = pyridoxine, folic acid, thiamin, Ca pantothenate and B_{12}
B vitamin solution III = solution II without thiamin and B_{12}
B vitamin solution IV = solution II without thiamin, B_{12} and Ca pantothenate
B vitamin solution V = pyridoxine

Since it has been shown that pyridoxine was a nutrient of the flukes, an experiment was carried out to see whether two related compounds of pyridoxine, pyridoxal and pyridoxamine will also show the same activity as pyridoxine in cultures. Using basal + A.A. + B vitamin solution V (pyridoxine) as controls the development attained when (a) pyridoxamine (B vitamin solution VII) and (b) pyridoxal (B vitamin solution VIa) was added to the basal + A.A. medium was investigated (Table 8). There was a change in the method of assessing media in this experiment. In previous experiments, media were assessed on the basis of the development attained by the best 10 flukes in the medium; the limitations of this method has been discussed in the introduction. The media in this experiment and all the rest to be described hereafter in this section were assessed on random sampling of 25 flukes from each culture and the final pH was measured immediately a culture was removed from the water bath.

Development in basal + A.A. + pyridoxamine medium was just as good as in the basal + A.A. + pyridoxine medium (Table 8) but all the flukes were found dead in the three cultures containing pyridoxal. Similar results were obtained supplementing basal + A.A. with another solution of pyridoxal (B vitamin solution VIb), pyridoxal being added to give a final concentration of 50 µg/ml. medium.

Table 8

Shows the effect of various forms of vitamin B_6 on the development of D. phoxini cultured in basal + amino acids medium.

Culture no.	Additive to basal + A.A.	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
58C1	B vit. sol. V (pyridoxine)	1	24	17	18	13	-	6	6.0
58C2		-	25	14	17	12	-	6	6.3
58C3		1	24	11	16	15	-	6	6.1
	PERCENTAGE	3	97	56	68	53	0		
58A1	B vit. sol.VII (pyridoxamine)	1	24	10	15	11	-	6	6.1
58A2		1	24	12	15	11	-	6	6.3
58A3		2	23	14	10	7	-	6	6.3
	PERCENTAGE	5	95	48	53	39	0		
58X1	B vit. sol.VIA (pyridoxal)			120 DEAD FLUKES				6	6.4
58X2				105 " "				6	6.5
58X3				90 " "				6	6.4

The problem of the apparent toxicity of pyridoxal in the medium was next investigated. Since pyridoxal has a very reactive carbonyl group and is known to react with amino acids (Robinson, 1951), it was thought that it might be combining with some amino acids in the medium to form products which were toxic to the flukes. The amino acid supplement was therefore omitted from the medium in experiment 63 and development compared when the basal medium was supplemented with pyridoxal and pyridoxine respectively. The vitamin was added to give a final concentration of 50 µg/ml. medium. Results are given in Table 9.

In the basal + pyridoxine medium, 17% of the flukes developed sperms and 11% vitellaria; but all the flukes were dead in each of the three cultures containing basal + pyridoxal. This indicated that the toxicity was not due to reactions with amino acids. Another possibility was that the concentration of pyridoxal in the medium was too high. To test this cultures were set up (experiment 83, Table 9) using pyridoxal at 2.0 µg/ml. concentration. Controls contained 50 µg/ml. pyridoxine (cf. experiment 58 C1-C3, Table 8).

There was hardly any difference in the level of development attained in the media suggesting that the flukes required a lower concentration of pyridoxal than pyridoxine in the medium.

Table 9

Shows the effect of various concentrations of vitamin B_6 on the development of *D. phoxini* cultured in basal or basal + amino acids.

Culture no.	Additive to basal medium	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
63C1	pyridoxine (50 µg/ml. medium)	13	10	2	2	2	-	6	6.6
63C2		5	20	5	4	3	-	6	6.4
63C3		10	14	6	2	2	-	6	6.5
	PERCENTAGE	37	59	17	11	9	0		
63X1	pyridoxal (50 µg/ml. medium)	ALL FLUKES DEAD						6	6.9
63X2								6	6.7
63X3								7	6.6
83X1	amino acids + pyridoxal (2.0 µg/ml.medium)	-	25	8	13	9	-	6	6.3
83X2		2	23	7	13	12	-	6	6.3
83X3		2	23	7	7	4	-	6	6.3
	PERCENTAGE	5	95	29	44	33	0		
83P2	amino acids + pyridoxine (50 µg/ml. medium)	2	23	10	11	10	-	6	6.2
83P3		3	22	11	13	9	1	6	6.3
	PERCENTAGE	10	90	42	48	38	2		

For the purpose of comparing various media, it was found desirable to repeat cultivation in some of the media which were previously assessed on the development attained by the best 10 flukes in the culture. These media were re-assessed by development attained by 25 flukes randomly selected from each culture the pH of cultivation being between the range 6.0-6.9. These results are shown in Table 10. Table 11 summarizes the results obtained with the various media described in this section.

Table 10

Shows the effect of adding (a) no supplement, (b) amino acids, (c) amino acids + B vitamins to a basal medium (BSSG + HS + alb).

Culture no.	Additive to basal medium	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
67X1	none	16	8	-	1	1	-	6	6.7
67X2		15	5	1	1	-	-	6	6.2
67X3		18	7	-	-	-	-	6	6.7
	PERCENTAGE	65	27	1	3	1	0		
67N2	amino acids	3	22	2	3	-	-	6	6.6
67N3		2	23	2	6	3	-	6	6.4
67T2		7	18	2	3	-	-	6	6.7
67T3		4	21	4	4	2	-	6	6.3
	PERCENTAGE	16	84	10	16	5	0		
91V1	amino acids + B vit. sol.Ia 0.02 ml./ml. medium	6	19	6	8	8	-	6	6.3
91V2		5	20	5	8	6	-	6	6.4
91V3		5	20	6	12	11	-	6	6.3
	PERCENTAGE	21	79	23	37	33	0		

Table 11

Shows the effect of adding yeast and B vitamins to basal (BSSG + HS + alb) with and without amino acids.

	Additive to BSSG + horse serum + albumen (basal medium)	No. of flukes / No. of cultures	Percentage of flukes developing genitalia					
			Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs
1	none	75/3 (Table 10)	65	27	1	3	1	0
2	amino acids	100/4 (Table 10)	16	84	11	16	5	0
3	pyridoxine (50 µg/ml.)	75/3 (Table 9)	37	59	17	11	9	0
4	amino acids + pyridoxine (50 µg/ml.)	125/5 (Tables 8 & 9)	6	94	50	60	47	1
5	amino acids + pyridoxal (2 µg/ml.)	75/3 (Table 9)	5	95	29	44	33	0
6	amino acids + pyridoxamine (50 µg/ml.)	75/3 (Table 8)	5	95	48	52	39	0
7	amino acids + B vit. sol. Ia	75/3 (Table 10)	21	79	23	37	33	0
8	yeast extract	225/9 (Table 39)	10	90	65	52	41	0
9	amino acids + yeast extract	150/6 (Table 25)	6	94	80	75	68	6

Discussion

Yeast extract has considerable growth promoting effects when added to a basal medium consisting of glucose balanced salt solution, horse serum and albumen (Wilson, 1960). A high percentage (45%) of yeast extract is amino acids (Sykes, 1956); and the importance of adding amino acids to a basal medium of glucose-saline, serum and albumen is shown in experiment 67 (medium 2, Table 11). A much higher percentage of the flukes form advanced testes and a small increase in the number of flukes producing sperms and vitellaria occurs. But this result is still far inferior to that obtained with yeast + basal medium, suggesting that yeast provides substances other than amino acids.

As yeast is rich in the B vitamin complex, (Sykes, 1956; Difco, 1960) attempts were made to test the effects of replacing yeast by a solution of synthetic B vitamins. Using a solution of vitamins recommended for tissue culture, Waymouth's medium 752/1 (quoted by Paul, 1959), containing nine vitamins, levels of development were compared when this solution (B vitamin solution I) and yeast were added separately to the basal + amino acid medium. Results obtained (Table 4) indicated that only part of the activity of yeast in the medium was replaced by the vitamins.

It was observed that whilst fairly contant results were obtained in culture containing yeast medium, variable results were obtained in the cultures in the vitamin cultures, some showing poor development. Two hypotheses were adduced to account for the poorer development.

1. Inadequate supply of some vitamins in the medium.

2. Yeast was supplying nutrient other than the B vitamins and amino acids added to the medium.

By doubling the concentration of vitamins previously used, using 0.05 ml./ml. medium instead of 0.025 ml., it was possible to reduce inter-culture variation and to obtain better results in the vitamin medium (Table 6). This result showed that the first hypothesis was at least in part right, and so at this stage the second was not investigated.

Having demonstrated the beneficial effects of the B vitamins, it was decided to try to elucidate the important vitamin in B vitamin solution I. Individual vitamins were gradually omitted from the solution and the activity of the solution tested until the most active vitamin was established. The scheme of elimination was based on the occurrence of B vitamins in albumen (Table 3), particular attention being paid to those

vitamins absent from or present in trace quantities. (Some B vitamins also occur in serum but no details of the quantity could be found.) Four different B vitamins solutions were prepared and the results when each was added to the basal + amino acid medium are shown in Table 7.

B vitamin solution II differs from the original vitamin solution (B vitamin solution I) by having an increase concentration of pyridoxine and by the absence of biotin, m-inositol, riboflavin and nicotinamide. Riboflavin and nicotinamide were excluded as they occur in larger quantities in albumen. Due to the fact that biotin is inactivated by egg-white in vitro (Robinson, 1951) it was felt that all the biotin in solution I was probably destroyed by albumen and hence it was left out in solution II. m-inositol was excluded (although no figures for its content in albumen were found) because it has been shown to be present in excess quantities in nearly all B vitamin rich natural products analysed. No loss of activity was observed when solution II instead of solution I was added to the basal + amino acid medium (Table 7); suggesting that biotin, m-inositol, nicotinamide and riboflavin were not the important B vitamins contributed by solution I.

It was decided to eliminate the remaining five vitamins

in solution II gradually, so as to determine the most active vitamin. Vitamin B_{12} was omitted along with thiamin from solution II to give solution III which contained pantothenic acid, folic acid and pyridoxine. Again, no loss of activity was observed when solution II was added to the basal + amino acid as compared with when solution I was added to the same medium (Table 7). (A difference of less than 20% was considered to be of little significance when cultures were assessed on the best 10 flukes.) Thiamin and vitamin B_{12} were therefore presumed to be present in adequate amount for the flukes in the basal medium.

Since the omission of pantothenic acid from solution III (forming solution IV) did not cause a significant change, it was assumed that pantothenic acid was not the active constituent of solution I. Folic acid and pyridoxine remained.

In solution V, folic acid was omitted. The results obtained using pyridoxine alone were as good as those obtained using solution I. It was concluded therefore that vitamin B_6 was the important part of the B vitamin supplement. An arbitrary further increase was made in the amount of pyridoxine in solution V so that its final concentration in the medium was 50 µg/ml. as compared with

20 µg/ml. when solutions II, III and IV were used and 2 µg/ml. when solution I was used. This high concentration did not have any adverse effects on the flukes but instead produced good results. It was not considered expedient at this stage to study the concentration of pyridoxine required by the flukes in cultures since the basal medium contained serum and albumen with varying vitamin content. Such problem can best be investigated in chemically defined media.

That yeast was contributing both pyridoxine and amino acids to the medium was apparent when pyridoxine and the amino acid supplement were added separately to the basal medium (Table 11). The addition of pyridoxine increased slightly the number of flukes producing sperms and vitellaria. Almost the same level of development was observed in the amino acid supplemented cultures But the synergistic effect on combining the two produced increases on the number of flukes producing sperms and vitellaria.

It was found that results obtained with various media which were previously assessed on the best 10 flukes in each culture were not invalidated when such media were re-assessed by random sampling. However, as the latter assessments were done on cultures maintained at a better

pH range (6.0-6.9), it was observed that there was an all round improvement in the level of development in the cultures.

Using pyridoxamine and pyridoxal instead of pyridoxine at a concentration of 50 µg/ml. of medium, it was discovered that the flukes showed reasonable development in the case of pyridoxamine, but died in this concentration of pyridoxal (Table 8 & 9). Pyridoxal from two sources gave similar results. This was unexpected as it is usually accepted in higher animals that the three forms of B_6 are interchangeable (Fruton & Simmonds, 1953). Pyridoxal at a concentration of about 2 µg per ml. medium gave the results obtained with pyridoxine at a concentration of 50 µg/ml. (Table 9).

Kidder & Dewey (1951) found pyridoxal and pyridoxamine to be approximately equal in activity and as much as 500 times as active as pyridoxine for the in vitro cultivation of the ciliate, Tetrahymena geleii. It is possible that with D. phoxini pyridoxal is the most active form of B_6 and hence is required at lower concentrations.

The finding that pyridoxine or its related compounds are nutrients for the flukes supports Chance and Dirnhuber's assumptions (see Introduction) that pyridoxine

is an essential substance in the metabolism of parasitic worms. Their assumptions were based on the occurrence of a higher concentration of pyridoxine in the worms than in the liver of their host.

However, although B vitamins + amino acids improve development considerably they do not give as good results as yeast extract. It would seem therefore that yeast contributes substances other than B vitamins and amino acids. Hence to learn more about these substances that promote growth, fractionation of the yeast was carried out as described in Section IV.

Summary

1. Yeast extract shows considerable growth promoting effects when added to a medium consisting of albumen, horse serum, glucose balanced salt solution and amino acids.

2. The activity of yeast in the medium is replaced partially by a solution of nine water soluble B vitamins.

3. A high concentration of pyridoxine or pyridoxamine completely replaces the effects of the other eight vitamins in the medium.

4. A low concentration of pyridoxal also replaces the effect of the other vitamins but at high concentration pyridoxal, unlike pyridoxine and pyridoxamine is toxic.

5. The addition of pyridoxine to the basal medium, i.e. glucose balanced salt solution, albumen and horse serum, promoted some development of genitalia and about 15% of the flukes produce sperms and 10% vitellaria. Further supplementation of the medium with amino acids results in an increase in the number of flukes producing sperms and vitellaria.

6. It is suggested that yeast helps development of the flukes by contributing amino acids and B_6 to the medium and probably some other growth factors, the effects of which can partly be replaced by a high concentration of pyridoxine.

SECTION II: THE REPLACEMENT OF YEAST EXTRACT BY LIVER EXTRACT IN CULTURE MEDIA

Introduction

Although a yeast medium was much more transparent than a yolky medium and therefore had the practical advantage of there being little difficulty in recovering the flukes from the cultures (Wilson, 1960), the activity of yolk was only partly replaced by yeast. It was considered worthwhile before applying analytical procedures to yeast, to try to replace yeast by a similar transparent extract of some natural products, in the hope of at least achieving the development attained in a yolk-albumen medium (Bell & Smyth, 1958). In selecting a suitable replacement for yeast, two factors were considered. Firstly, the extract must supply, amongst other nutrients a large amount of amino acids comparable to that present in yeast, and secondly, it should be rich in pyridoxine.

Liver was selected because of its good protein content, viz. 190 mg./gm rat fresh tissue (Spector, 1956); its high pyridoxine content (1.7 g-12 µg/gm) depending on the animal (Robinson, 1951), and because extracts of liver prepared with various solvents are transparent. Liver extract have been incorporated in many media for the *in vitro* cultivation of helminths. For example

Weinstein & Jones (1956) used it as a medium constituent when culturing the histotrophic stages of Nippostrongylus muris; Silverman (1959) for the cultivation of Haemonchus contortus and Ostertagia spp., and Dougherty (1953) for culturing the free living nematode, Rhabditis briggsae, to mention a few.

However, Wilson (1960) failed to get an improved level of development in D. phoxini when raw mouse liver extract was added to a basal medium consisting of balanced salt solution, horse serum and albumen. It was decided to repeat Wilson's experiments with slight modification, and in this section the activity of liver extract prepared from liver of different animals using water and other organic solvents is described. The effects of ageing on such extract was also studied.

Materials and Methods

1. Raw liver extract (RLE)

Liver (about 10 gm) from a pigeon killed by exsanguination, was removed promptly and homogenised in a Nelco blender in 40 ml. distilled water at 4°C for 10 minutes. The homogenate was centrifuged at about 3,000 g for 10 minutes. The supernatant pipetted off and sterilised by seitz filtration. 1 ml. of the extract was added to 9 ml. basal medium. Only freshly prepared extracts

were used.

2. Autoclaved water extract of liver (ALE)

Two methods of preparation were used.

Method 1

A similar homogenate as that used for RLE was autoclaved at 15 lbs/sq.in. for 15 minutes. Some of the proteins of the liver were precipitated by heat; the clear fluid was decanted from the protein clot, dispensed in 10 ml. aliquots in universal containers and re-autoclaved, cooled and stored at 4°C. Extracts using rat, pigeon, duck and horse liver, respectively, were prepared.

Method 2

Pieces of pigeon liver were allowed to undergo autolysis before the extract was prepared.

The liver (about 10 gm) was removed from a pigeon and left exposed to the atmosphere at room temperature in a petri dish for eight hours. After this period it was homogenised in 40 ml. distilled water and left to stand overnight in a stoppered 125 ml. bottle. The homogenate was centrifuged the following morning, and the supernatant autoclaved at 15 lbs/sq.in. for 15 minutes. The clear fluid was decanted from the protein clot, dispensed in 10 ml. aliquots in universal containers and

re-sterilised by autoclaving. 1 ml. of the autoclaved extract, prepared by either method was added to 9 ml. of basal medium.

3. Ethanol extract of liver (EEL)

About 10 gm of liver was homogenised in 40 ml. of ethanol for 10 minutes. This was left standing overnight, centrifuged the following day at 3,000 g for 10 minutes and the supernatant pipetted off. The volume of the supernatant was reduced to about 5 ml. by distillation under reduced pressure at between 30-40°C. The resulting oily liquid was sterilised by autoclaving. 5 ml. of the autoclaved extract was dissolved in 5% aqueous Tween 80 (5 ml.), diluted with 90 ml. sterile distilled water and dispensed in 10 ml. portions in universal containers. 1 ml. of this extract was added to 9 ml. basal medium.

4. Butanol extract of liver (BEL)

About 10 gm of fresh liver was homogenised with 40 ml. butanol for 10 minutes. The homogenate centrifuged at about 3,000 g for 10 minutes, the supernatant pipetted off, distributed in 10 ml. portions in universal containers and sterilised by autoclaving. 1 ml. of the extract was added to 9 ml. basal medium.

5. Butanol extracted, ethanolic liver extract (BEELE)

Due to the fact that the butanolic extract was insoluble in water and also in aqueous Tween 80, it had to be evaporated to dryness, re-dissolved in ethanol before it could be diluted with Tween 80.

About 30 ml. of the supernatant in (4) was placed in a crystallising dish covered with a watch glass and evaporated to dryness. The resulting product was transferred to a universal container, dissolved in 2 ml. ethanol and left standing overnight to render it sterile. This was next pipetted into a 125 ml. bottle, 10 ml. of aqueous Tween 80 added and the volume made up to 100 ml. with distilled water. 1 ml. of this extract was added to 9 ml. basal medium.

Results

Firstly, the growth promoting effects of water and butanolic extracts of pigeon liver were demonstrated by supplementing the basal medium with ALE, BEL and BEELE, respectively; the basal medium used consisted of horse serum, albumen and BSSG (Table 12).

Autoclaved liver extract (ALE) showed considerable growth properties when added to the basal medium; as much as 80% of the selected flukes developed vitellaria but only half of these had active sperms. (cf. cultures 44A1-A3 and cultures 44W2-W3.)

The undiluted extract (BEL) introduced some toxicity into the medium as the flukes were all dead in the basal medium supplemented with it (cultures 41D1 and D3).

The diluted butanolic extract (BEELE) did not seem to possess any growth promoting quality as it did not stimulate further development on addition to the basal medium.

Table 12

Development in basal medium (BSSG + HS + alb) supplemented with various liver extracts.

Culture no.	Liver extract added to basal medium	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Fin pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
44A1	none	10	1	1	2	-	-	-	-	8	30	6	7.3
44A2		10	-	-	2	1	1	-	-	10	51	6	7.3
44A3		10	2	2	-	-	-	-	-	10	59	6	7.3
	PERCENTAGE	100	10	10	13	3	3	0	0				
44W2	autoclaved (ALE)	10	6	2	10	7	7	7	-	10	56	6	6.8
44W3		10	6	4	10	9	9	7	-	10	55	6	6.8
	PERCENTAGE	100	60	30	100	80	80	70	0				
44K1	Butanol Ethanol diluted (BEELE)	10	-	-	-	-	-	-	-	8	30	6	6.9
44K2		10	1	1	-	-	-	-	-	8	25	6	6.8
	PERCENTAGE	100	5	5	0	0	0	0	0				
44D1 44D3	Butanol (BEL)	FLUKES ALL DEAD											

Table 13 compares level of development in the basal medium supplemented with autoclaved, water liver extract (ALE) and diluted ethanolic liver extract (EEL).

The general level of development in the EEL supplemented medium was only the development of testes (cultures 46K1 and K3). Whilst sperms, vitellaria and yolk reservoirs were found in over 70% of the selected flukes in the ALE supplemented cultures. It appears therefore that water was the most suitable solvent for preparing active extracts of liver. This conclusion was further substantiated when a repeat experiment (experiment 107 E1-E3, Table 14) gave similar results.

Table 13

A comparison of the level of development attained when basal medium (BSSG + HS + alb) is supplemented with autoclaved water extract of liver (ALE) and ethanolic liver extract (EEL).

Culture no.	Liver extract added to basal 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	Vitel-:laria	Yolk reser-:voir	Eggs				
46Z1	autoclaved (ALE)	10	8	5	10	8	10	10	-	12	60	6	6.8
46Z2		10	7	3	10	10	10	10	-	12	70	6	6.4
46Z3		10	8	2	10	6	7	5	-	10	58	6	6.7
	PERCENTAGE	100	77	33	100	80	90	83	0				
46K1	Ethanol (EEL)	10	-	-	-	-	-	-	-	8	45	6	6.9
46K2		10	1	-	1	-	1	-	-	8	30	6	6.8
	PERCENTAGE	100	5	0	5	0	5	0	0				

As water had been found to be the most suitable solvent for the extracting of some of the growth factors of liver, the level of development attained in using raw liver extract (RLE) and autoclaved liver extract (ALE) were next compared with development in yeast; the growth effects produced by these extract on addition to the basal medium are shown in Table 14. These media were assessed by random sampling and cultivation was carried out at pH 6.3-6.9.

Development in the ALE supplemented medium was better than in the yeast or RLE supplemented media. Sperm production in the ALE medium was almost identical with that obtained in the yeast medium; but vitellaria production was poorer in the latter medium than in the former. It should be added that the yeast controls were below average for this medium. However, ALE appears to be at least as effective as yeast and better than RLE (cultures 107 L1-L3).

Table 14

Shows the effect of adding autoclaved liver extract (ALE), raw liver extract (RLE), ethanolic liver extract (EEL) and yeast extract to basal medium (BSSG + HS + alb).

Culture no.	Additive to basal medium	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
107A1	ALE	2	23	17	16	13	-	6	6.4
107A2		2	23	13	13	10	-	6	6.3
107A3		-	25	17	15	12	-	6	6.5
	PERCENTAGE	5	95	63	59	47	0		
107L1	RLE	2	23	15	10	6	-	6	6.4
107L2		3	22	9	7	4	-	6	6.3
107L3		3	22	12	11	7	-	6	6.5
	PERCENTAGE	11	89	48	37	23	0		
107E1	EEL	15	10	2	1	-	-	7	6.5
107E2		18	7	1	-	-	-	7	6.3
107E3		12	13	-	-	-	-	7	6.4
	PERCENTAGE	60	40	4	1	0	0		
107E1	yeast extract	2	23	15	9	7	-	6	6.5
107E2		3	22	12	10	10	0	6	6.4
	PERCENTAGE	10	90	54	36	34	0		

All the foregoing experiments were carried out with extracts of liver obtained from a laboratory host of D. phoxini, the pigeon. In Table 15, the development attained with ALE prepared from rat, duck and horse liver is compared with that obtained with ALE prepared from pigeon.

There appeared to be no difference in the level of development attained in the basal medium supplemented with the extracts prepared from the different animals, and they all confirm the value of autoclaved liver extract as a medium constituent for culturing D. phoxini.

Table 15

The effect of supplementing the basal medium (BSSG + HS + alb) with autoclaved liver extracts (ALE) prepared from horses', rats', ducks' and pigeons' liver.

Culture no.	Liver extract added to basal medium	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
62C1	ALE (pigeons')	1	24	15	11	5	-	6	6.6
62C2		2	23	11	15	14	-	6	6.1
62C3		-	25	16	15	13	-	6	6.4
	PERCENTAGE	4	96	56	55	43	0		
68K1	ALE (horses')	-	25	18	13	11	2	6	6.5
68K2		2	23	18	18	15	-	6	6.6
	PERCENTAGE	4	96	72	62	52	4		
91D1	ALE (ducks')	1	24	16	10	9	-	6	6.5
91D2		-	25	12	11	11	3	6	6.5
91D3		-	25	13	11	8	-	6	6.5
	PERCENTAGE	1	99	55	41	37	4		
91R1	ALE (rats')	1	24	12	11	9	1	6	6.4
91R2		2	22	11	12	11	-	6	6.4
91R3		-	25	9	13	12	2	6	6.4
	PERCENTAGE	4	96	43	48	43	4		

Table 16 shows development in the ALE + basal medium, in which the ALE was (a) freshly prepared, (b) stored for a month (c) prepared from liver which had undergone autolysis.

There was no difference between the three sets of cultures; which suggests that the important metabolites in the extract are not lost on storage, nor degraded by autolysis.

Table 17 shows in summary the level of development reached in basal alone, in basal + yeast, and in basal + autoclaved liver (experiment 62, Table 15; experiment 61, Table 34, experiment 107, Table 14).

Table 16

Development in basal medium (BSSG + HS + alb) supplemented with different autoclaved liver extracts.

Experiment no.	Additive to basal medium	Percentage of flukes developing genitalia					
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs
107 (Table 14)	ALE (freshly prepared)	5	95	63	59	47	0
61 (Table 34)	ALE (1 month old)	7	93	56	56	51	1
62 (Table 15)	ALE (autolysed liver)	4	96	56	55	43	0

Table 17

A comparison of development in basal + autoclaved liver extract (ALE) and basal + yeast extract.

No. of flukes / No. of culture	Additive to basal (BSSG + HS + alb)	Percentage of flukes developing genitalia					
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs
75/3 (Table 10)	none	65	27	1	3	1	0
225/9 (Tables 14, 15 & 34)	ALE (autoclaved liver extract)	4	96	58	56	47	0
225/9 (Table 39)	YE (autoclaved yeast extract)	10	90	65	52	41	0

Discussion

The idea that an extract of liver would be an effective supplement for the *in vitro* cultivation of *D. phoxini* developed from the results of the previous section, in which it was found that some form of vitamin B_6 and amino acids were essential nutrients of the flukes. As was mentioned in the "Introduction", liver is rich in B_6 and has a high protein content. Early experiments were carried out using liver from a good laboratory host of *D. phoxini*, the pigeon, as Wilson (1960) failed to improve development by supplementing a medium with raw liver extract of mouse (a poor laboratory host). Later it was found that extracts prepared from the liver of rat and horse, which are not as far as is known possible hosts, were just as effective as those from duck and pigeon (Table 15).

Autoclaved liver extract (ALE) showed considerable growth promoting activity when added to the basal medium; but such activity was only given by extracts prepared with water. Diluted ethanol and butanol extracts were found to be ineffective supplements (Tables 13 and 14). Although these organic solvents had advantages over water in that they were good solvents for fatty substances, fat soluble vitamins, certain amino acids and B vitamins,

they could not be used without aqueous dilution as even one drop per culture introduced a toxic level of alcohol. The inactivity of the diluted alcoholic extracts might be due to the fact that these contain less amino acids and pyridoxine than the water extracts. The solubility of some of the amino acids and pyridoxine is much lower in alcohol than in water, e.g. l and d cystine and dl histidine are soluble in water but insoluble in alcohol; the solubilities of glycine, isoleucine and valine in 100 ml. water at 25°C are 25.3, 5.02 and 7.44, respectively, as compared with 0.043, 0.157 and 0.571, respectively in 100 ml. ethanol at the same temperature. Pyridoxine has a solubility of 1.1 in 100 ml. alcohol as compared with 22.0 in 100 ml. water (Hodgman, 1947).

Autoclaved liver extract (ALE) seemed to be more effective than raw liver extract (RLE) (Table 14). This might be due to the fact that the activity of vitamin B_6 increases on autoclaving (Robinson, 1951). Kidder & Dewey (1951) found that the ciliate, Tetrahymena geleii, required 0.45 µg per ml. of pyridoxine in vitro when sterilised by filtration or 0.25 µg per ml. when the medium was sterilised by autoclaving; the activity of the pyridoxine being increased on heating with amino acids. An alternate explanation is that one or more of the heat labile substances is toxic, and that this toxicity

is lost by autoclaving.

There was no difference in activity in ALE which was prepared from fresh liver and that obtained from one which had undergone autolysis, this suggests that the important metabolites in the extract are not degraded by autolysis.

There was no change in activity in ALE which had been stored at about 4°C for one month (Table 16). The ageing effect was investigated as Dougherty (1953) found that a liver protein fraction became active as a nutrient for the nematode, Rhabditis briggsae, after storage for two months.

All that can be concluded is that a clear aqueous extract can be prepared from liver of various species of animals, which need not be potential hosts, and that providing the extract is autoclaved (heated) it gives as good a result as that obtained with yeast extract. However, it does not support development nearly as well as yolk. At this stage of the work it had to be decided whether to continue with liver extract media or return to yeast supplemented media. As a considerable amount was already known about the effect of different fractions of yeast and as liver did not appear to be appreciably better, it was decided to postpone a further investigation of the liver extract and to try to get improved development in yeast.

Summary

1. Water liver extract shows considerable growth promoting effects when added to a basal medium consisting of balanced salt solution, horse serum and albumen.

2. Butanolic or ethanolic extracts are not effective supplements.

3. The activity of the water extract increases on autoclaving.

4. Extracts prepared from horses' and rats' (not hosts of D. phoxini) are as effective as those from ducks' and pigeons' (host of D. phoxini) liver.

5. The active components of the extract is not destroyed by autolysis or by storage at $4^{o}C$ for one month.

6. Development in the basal medium supplemented with autoclaved liver extract is as good as that obtained in the basal + yeast medium.

SECTION III: THE SUPPLEMENTATION OF THE YEAST MEDIUM WITH EMBRYONIC MATERIALS

Introduction

Bell & Smyth (1958) using yolk and albumen succeeded in obtaining nearly complete maturation of D. phoxini. The only qualitative defects were the abnormal staining reaction of the vitellaria and the failure to form normal egg-shell. The replacement of yolk by yeast plus horse serum resulted, however, in the reduction of the number of flukes producing eggs. It seemed, therefore, that some growth factors promoting normal development of the flukes were present in yolk, but absent, or in inadequate amounts in the yeast medium.

It was decided to supplement the yeast medium with some embryonic materials to test whether these would improve development in the medium. The medium was supplemented with aqueous chick embryo extract and with some fat soluble fractions of hen egg yolk. Hen egg yolk is known to be rich in lipids. These lipids though complex in nature contained true fats, phospholipids and sterols. Of the sterols found in yolk, cholesterol appeared to be the most important, 84% of its total quantity being found in the free state (Romanoff & Romanoff, 1949). Development was studied in the yeast

medium supplemented with egg-lecithin, cholesterol and oleic acid.

Alpha-tocopherol was used as a supplement as it is found in the non-lecithin fraction of lipids extracted from yolk (Serono & Monezemolo, 1943).

Materials and Methods

1. Embryo extract

Embryo extract was prepared as described in Section V. It was used at a final concentration of 4% in the medium.

2. Egg-lecithin

Egg-lecithin was used both as a suspension and as a solution.

(a) Suspension of lecithin in BSSG

0.84 gm of egg-lecithin obtained from Nutritional Biochemical Corporation, Ohio, was sterilised by dissolving in 3 ml. absolute alcohol in a sterile petri dish. The alcohol was left to evaporate under the ultra-violet lamp in the sterile cabinet. The sterile product was transferred to a universal container, and 10 ml. of BSSG were added. The mixture was shaken to give a suspension.

(b) A solution of lecithin in oleic acid

0.42 gm of egg-lecithin was dissolved in 3 ml. alcohol in a 125 ml. bottle, and left standing for about 2 hours. This was diluted with 10 ml. of a 5% sterile aqueous Tween 80 (oleic acid) and the volume made up to 100 ml. with sterile distilled water. This was shaken in a mechanical shaker for 10 minutes and then stored at 4°C. 0.5 ml. of this solution was added to 11-12 ml. basal + yeast medium. 1.5 ml. of a suspension of egg-lecithin in BSSG was added to 11-12 ml. basal + yeast medium.

3. Cholesterol

Cholesterol was used as a suspension. 4.0 mg cholesterol obtained from B.D.H. were suspended in 10 ml. distilled water in a universal container. A few glass beads were added and the whole autoclaved at 15 lbs/sq.in. for 15 minutes. This was cooled and shaken to give a fine suspension of cholesterol in water. 0.4 ml. of the suspension was added to 11-12 ml. basal + yeast medium.

4. Alpha-tocopherol

Alpha-tocopherol was used as a suspension in BSSG and also as a solution in oleic acid.

Suspension of alpha-tocopherol in BSSG

100 mg of d-alpha-tocopheryl acid succinate from N.B., Co., Ohio, were sterilised as described in (2b). The sterile product was suspended in 25 ml. BSSG to give a suspension. 0.1 ml. of the suspension was added to 11-12 ml. basal + yeast medium.

Solution of alpha-tocopherol in oleic acid

The method used was similar to that described for lecithin in (2). Several solutions using either d-alpha-tocopheryl acid succinate or dl-alpha-tocopheryl acetate at different concentrations were prepared.

5. Supplementation of the yeast medium with thioglycollic acid

Five milligrams of yeast extract were dissolved in 28 ml. BSS; to this was added 0.5 ml. thioglycollic acid (B.D.H.). A small pellet of NaOH was added to bring the pH to 6.9 before the solution was autoclaved at 110°C for 15 minutes. The solution was cooled, 10 ml. of a 6% glucose solution and 16 ml. of horse serum added, and dispensed in 10 ml. portions in universal containers. 1.5 ml. amino acid, 1.5 ml. of albumen, 1,000 units of penicillin and 0.25 mg streptomycin were added to each

tube and the pH adjusted to 6.9.

6. Yeast extract

The yeast extract was prepared as described in Section I.

7. Basal medium

The basal medium consisted of glucose balanced salt solution (BSSG); horse serum, (30%), HS; (12½%) albumen (alb); and amino acids (AA).

Results

Firstly, the effects of using chick embryo extract as a supplement to the yeast medium (basal + yeast extract) was studied. 0.8 ml. of embryo extract (prepared as described in Section V) was added to 10-12 ml. of the medium (Table 18).

The embryo extract did not seem to improve the growth promoting qualities of the medium; suggesting that it was not contributing any additional nutrient for the flukes to the medium.

Table 18

Development in the yeast medium supplemented with chick embryo extract.

Culture no.	Additive to yeast medium (basal + yeast)	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
92B1	none	-	25	19	14	14	2	6	6.6
92B2		2	23	22	16	12	-	6	6.4
	PERCENTAGE	4	96	82	60	52	4		
92E1	embryo extract	-	25	14	15	14	-	6	6.4
92E2		1	24	13	14	11	-	6	6.5
92E3		-	25	16	13	13	-	6	6.4
	PERCENTAGE	1	99	57	56	51	0		

As the yolk of a hen egg is rich in lipids, egg-lecithin was used as a supplement to the yeast medium (basal + yeast extract). The effects of adding cholesterol to the lecithin supplemented medium was studied as this compound is also abundant in a free state in yolk (Romanoff & Romanoff, 1949). Both lecithin and cholesterol were used as suspension in balanced salt solution. The results are shown in Table 19.

Using cultures containing the yeast medium as controls (cultures 39C1-C2), the addition of lecithin (cultures 39L1-L3), and lecithin and cholesterol (cultures 39H1-H2) to the yeast medium did not produce better results than those obtained in the controls (39C1-C2).

Table 19

Development in the yeast medium supplemented with lecithin and lecithin + cholesterol.

Culture no.	Additive to yeast medium (basal + yeast)	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Fina pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
39C1	none	10	8	7	10	10	10	10	1	10	48	6	7.0
39C2		10	7	7	10	10	10	10	-	10	54	5	7.0
	PERCENTAGE	100	75	70	100	100	100	100	5				
39H1	suspensions of lecithin + cholesterol	10	8	3	10	5	3	-	-	10	61	6	7.0
39H2		10	6	6	7	5	3	3	-	10	60	5	7.0
	PERCENTAGE	100	70	45	85	50	30	15	0				
39L1	suspension of lecithin	10	6	6	7	5	3	3	-	10	51	6	7.1
39L2		10	5	4	10	4	3	3	-	8	34	6	7.1
39L3		10	6	5	10	5	4	2	-	10	55	5	7.0
	PERCENTAGE	100	57	50	90	47	33	27					

It was thought that the failure to improve development of the flukes might be due to the lecithin and cholesterol being added in an unsuitable form. The experiment was repeated using the lecithin as a solution in oleic acid (cultures 42L1-L3; Table 20). It was also considered necessary to test components of the non-lecithin fraction of lipids extracted from yolk; one of such components is vitamin E (Serono & Montezemolo, 1943). Three closely related biological active compounds are known to comprise vitamin E, viz. alpha-, beta- and gamma-tocopherol (Finar, 1956, p.657). Using the yeast medium as controls, the effects of adding d-alpha-tocopherol as a solution in oleic acid at a final concentration of 2 µg/ml. in the medium was studied (Table 20).

The addition of lecithin to the yeast medium did not seem to produce any beneficial effect on the medium. Compare cultures 42L1-L3 and 42C1-C3. But on the other hand, the addition of d-alpha-tocopherol to the medium resulted in an increase of the number of flukes producing eggs. In culture 42E1, there were 7 flukes picked up with eggs in the uterus. Although tanning of egg-shell material occurred in localised areas of the egg, most of the eggs, however, seemed to possess the shape of a normal egg. There was also in all the alpha-tocopherol supplemented cultures a number of eggs in the medium;

few of them of a normal shape. These eggs had no shell and their shapes were greatly altered on fixation.

Table 20

The effect of adding (a) lecithin, (b) alpha-tocopherol to the yeast medium.

Culture no.	Additive to yeast medium (basal + yeast)	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Fina pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
42C1	none	10	10	7	10	10	10	10	-	10	-	6	6.5
42C2		10	10	10	10	10	10	10	-	10	40	6	6.4
42C3		10	10	10	10	10	9	7	-	10	40	6	6.3
	PERCENTAGE	100	100	90	100	100	97	90	0				
42L1	lecithin solution	10	10	10	10	10	10	10	-	10	50	6	6.3
42L2		10	10	10	10	10	10	10	-	10	40	6	6.3
42L3		10	10	10	10	10	10	10	-	6	20	6	6.7
	PERCENTAGE	100	100	100	100	100	100	100	0				
42E1	alpha-tocopherol	10	10	10	10	10	10	10	7	10	50	6	6.3
42E2		10	10	10	10	10	10	10	2	10	50	6	6.2
42E3		10	10	10	10	10	10	10	3	10	45	6	6.2
	PERCENTAGE	100	100	100	100	100	100	100	40				

The d-alpha-tocopherol used in the previous experiment was prepared by diluting its alcoholic solution with aqueous oleic acid (see Methods). As oleic acid was also present in yolk, an experiment was designed to show that the activity of the solution was due to the tocopherol. d-alpha-tocopherol was added to the yeast medium as a suspension in BSS; development in this medium was compared with that of the yeast medium supplemented with alpha-tocopherol prepared as a solution in oleic acid; using as controls, cultures containing the yeast medium (Table 21).

In the controls, 2 flukes had one egg each in their uterus (culture 40C3), and 1 fluke had an egg in culture 40C1, but no egg production was observed in culture 40C3. No eggs were found in the culture medium. In contrast to this there were about 20-40 eggs picked up from each of the alpha-tocopherol supplemented cultures. There appeared to be very little difference in the egg production of the flukes when alpha-tocopherol was used as a suspension in BSS or as a solution in oleic acid, showing that alpha-tocopherol rather than oleic acid was responsible for the increased egg production in the medium.

Development in culture 40Z3 was very good. 9 flukes out of the 10 examined had eggs in their uterus,

one of which had its posterior lobe almost completely filled with eggs. More than twelve eggs were counted in this flukes. The others had one to four eggs each.

Table 21

Development in the yeast medium supplemented with alpha-tocopherol.

Culture no.	Additive to yeast medium (basal + 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	Vitel-:laria	Yolk reser-:voir	Eggs				
40C1	none	10	9	9	10	10	10	10	1	10	60	5	7.1
40C2		10	8	8	10	10	10	10	-	10	50	6	6.8
40C3		10	7	9	10	10	10	10	2	10	40	6	6.8
	PERCENTAGE	100	87	87	100	100	100	100	10				
40Z1	alpha-tocopherol in oleic acid	10	10	10	10	10	10	10	2	10	40	6	6.3
40Z2		10	10	10	10	10	10	10	3	10	50	6	6.3
40Z3		10	10	10	10	10	10	10	9	10	40	6	6.7
	PERCENTAGE	100	100	100	100	100	100	100	47				
40X1	suspension of alpha-tocopherol in BSSG	10	10	10	10	10	10	10	6	12	60	5	7.0
40X3		10	10	10	10	10	10	10	1	10	50	6	7.1
	PERCENTAGE	100	100	100	100	100	100	100	35				

It was decided to test whether the dl-form was equally or more active than the d-form previously used. Development was studied in the yeast medium supplemented with d-alpha-tocopheryl acid succinate and dl-alpha-tocopheryl acetate respectively (Table 22). Only egg production was recorded. Each compound was supplied to give a final concentration of 50 µg/ml. in the medium.

There appeared to be no difference in the activity of the two compounds in the medium.

Table 22

Culture no.	Additive to yeast medium	No. of flukes with eggs	No. of eggs in medium
53T1	d-alpha tocopheryl succinate	5	42
53T2		1	12
53D1	dl-alpha tocopheryl acetate	2	27
53D2		2	16
53D3		-	20

As alpha-tocopherol is known to posses reducing properties, the activity of thioglycollic acid, another compound with reducing properties though not found in yolk, was tested by supplementing the yeast medium with it. The medium was prepared as described in the methods. Thioglycollic acid constituting less than 0.5% of the final medium.

The results were as follows:-

The flukes in three cultures containing yeast + thioglycollic acid medium were all found dead on examination on the sixth day. The pH of each culture was 6.3 and no bacterial infection was detected. This suggests that thioglycollic acid was toxic to the flukes.

Discussion

From the results of the foregoing experiments it appears that the failure of many of the flukes to produce eggs in the yeast medium was partially due to an inadequate supply of some nutrients in the medium. Supplementing the medium with chick embryo did not improve development, which would probably mean that the missing nutrients were not present in embryo extract (Table 18). This does not mean that embryo extract contains no suitable nutrient for D. phoxini on the contrary it was found later (Section V) that embryo extract was a suitable replacement for serum in the medium. It does suggest, however, that embryo extract like serum lacks certain substances essential for egg production in D. phoxini.

As lipids constitute 33% of hen egg yolk (Romanoff & Romanoff, 1949) the effects of supplementing the yeast medium with egg-lecithin, a complex component of the lipids of yolk, and with cholesterol, a sterol found mostly in the free state in yolk was studied. Neither lecithin nor cholesterol improved the egg productivity of the medium (Table 19). It was later realised that the amount of cholesterol added to the medium was small compared to the amount present in it already. In each culture, there were already about 2 mg of cholesterol present in

the serum, and therefore it was to be expected that a further addition of 0.16 mg to each culture would not produce any beneficial effects in the medium. Although information about the value of lecithin in horse serum was not available, it is possible that a similar explanation could be given for the failure of the low concentration of lecithin to stimulate further development in the medium.

Supplementing the yeast medium with alpha-tocopherol resulted in an increase of egg production as well as an improvement on the shape of the eggs; these were still abnormal and had no proper shell. It would appear that the yeast medium was deficient in some nutrients of which the effects can be replaced partially by alpha-tocopherol supplied either as a suspension in the medium or better still as a solution in oleic acid. There also appeared to be no advantage in using the racemic form instead of the dextro-rotatory form of alpha-tocopherol; both forms were equally active (Table 22).

As alpha-tocopherol possesses reducing properties (Spector, 1956), it makes it difficult to specify its role in cultures. It may be either a nutrient for the flukes, or an antioxidant altering the physical conditions of the medium. No attempt was made to study the effects which alpha-tocopherol had on the redox potential of the

medium as Wilson (1960) had already enumerated the technical difficulties involved in measuring the potential of a medium containing serum and albumen. Instead, however, thioglycollic acid, another antioxidant was added as a supplement to the yeast medium to test whether it would produce similar effects. This compound was, however, found to be toxic to the flukes.

The role of alpha-tocopherol in cultures could more easily be determined if a successful medium containing only defined components should be developed.

Summary

1. Alpha-tocopherol increases egg production when added to a medium consisting of glucose balanced salt solution, horse serum, albumen, amino acid and yeast extract.

2. Both dextro-rotatory and racemic forms of alpha-tocopherol were found to possess activity.

3. Embryo extract, egg-lecithin and cholesterol were found to be ineffective supplements.

4. Thioglycollic acid at a level of 0.5% in the medium was toxic to the flukes.

5. The probable role of alpha-tocopherol in the medium is discussed.

SECTION IV: YEAST EXTRACT AS A NUTRIENT IN CULTURE MEDIA

Introduction

In tissue culture, the problem of replacing natural substances in culture media by defined components was partially solved in two ways, using (a) the analytical and (b) the synthetic approaches (Paul, 1959). In the first approach, the medium was analysed and its essential components identified, whilst in the synthetic approach, metabolites which were shown to be essential for the development and maintenance of the intact animal were combined to form media which were then tested for their ability to keep cells alive and to stimulate growth.

The analytical method was mainly used in investigating the nutritional requirements of D. phoxini in vitro. The natural components in the medium, viz. horse serum, albumen and yeast were each subjected to a series of analytical procedures. In this section, the various analytical procedures applied to yeast extract, and their effects on the activity of the yeast extract in cultures are described.

That yeast extract shows considerable growth activity when added to a basal medium consisting of glucose balanced salt solution, horse serum and albumen

was shown by Wilson (1960). She also showed that the growth factors of yeast are thermostable and that dialysed yeast also possesses some growth stimulating factors. In Section I, in which an empirical approach used to replace yeast was described, it was shown that yeast could be replaced partially by pyridoxine and amino acids. It was also suggested that yeast was probably contributing other factors apart from vitamins and amino acids to the medium. It was hoped that, by applying analytical procedures to the yeast extract, it would be possible to isolate the required metabolites as well as to confirm results obtained in Section I, i.e. that yeast was contributing pyridoxine and amino acids to the medium.

Materials and Methods

Media

Basal medium

The basal medium used consisted of balanced salt solution, glucose, horse serum and albumen prepared as described in Section I (see Methods).

The amino acid supplement was that of Parker's medium 703 (Healy et al., 1954).

Analytical procedures

(1) Alkaline hydrolysis of yeast

Alkaline hydrolysis was carried out by autoclaving

a 4% yeast extract made up in 0.1 N NaOH for 15 minutes at 115°C. 5 ml. was added to 45 ml. gluco-saline and serum, the pH adjusted to 6-7 with 0.2 N HCl. The medium was dispensed in 10 ml. aliquots; 1.0-1.5 ml. of albumen was added to each culture and the pH adjusted to 6.9.

(2) Acid hydrolysis of yeast

Acid hydrolysis was carried out by refluxing 12% yeast extract in N HCl for one hour. The solution was then autoclaved at 115°C for 15 minutes. 1.7 ml. of the yeast was added to 45 ml. of basal medium without albumen. The pH was corrected to between 6 and 7 with 0.2 N NaOH, and the medium dispensed in 10 ml. aliquots.

(3) Dialysis of yeast

First method

One hundred millilitres of a 12% yeast extract were placed in a sterile one inch cellulose tube and dialysed against running water for 96 hours. The resulting solution was sterilised by autoclaving at 115°C for 15 minutes. 1.3 ml. of the sterile dialysed yeast extract was added to 10 ml. basal medium.

Second method

Dialysis was carried out by placing 50 ml. of an autoclaved 4% yeast extract in a sterile one inch diameter cellulose tube, and dialysed against running water for seven days. During this process the volume increased to 80 ml. The original volume was regained by evaporating under reduced pressure at 40-50°C the solution re-sterilised by autoclaving, dispensed in 10 ml. aliquots and stored at 4°C. 1 ml. of this solution was added to cultures.

(4) Dialysed hydrolysed yeast

Combined hydrolysis and dialysis consisted of refluxing a 12% solution as in procedure 2, diluting to 4% with distilled water and the dialysing, concentrating, sterilising and dispensing as in procedure 3 (second method). 1 ml. was added to each culture.

(5) Yeast extract through anion exchange column

Anion exchange was carried out using Dowex I resin in the chloride form. A six by one centimetre column (figure 5) was washed with N HCl to charge. Excess Cl^- was washed out with distilled water until no precipitate was formed with $AgNO_3$. 20 ml. of 4% yeast extract corrected with 0.2 N NaOH to pH 8

was passed through the column. The effluent was collected without washing the column and autoclaved. Similar procedures were also applied to a 2% yeast extract. 2 ml. and 1 ml. of the 2% and 4% effluents respectively were used per culture.

(6) Yeast extract through cation exchange column

For cation exchange, Zeo Karb 215 in the hydrogen form was used at pH 4. The resin was kept in 4 N HCl for 3 hours to convert to the hydrogen form, then washed with distilled water until the effluent was free of chloride ions. 20 ml. of 2% yeast extract was acid hydrolysed (procedure 2) pH corrected to 4 and passed through the column. The effluent was autoclaved, pH adjusted to between 6 and 7, and 2 ml. added to the basal medium in each culture.

Spot test for the detection of vitamin B_6

Vitamin B_6 is known to give a brownish-red colouration with ferric chloride (Stevens & Keresztesy, 1938). A solution of ferric chloride was prepared by dissolving 0.3 gm anhydrous $FeCl_3$ in 20 ml. distilled water.

Fifteen millilitres of an effluent through Dowex I were concentrated to about 2.5 ml. by distillation of the water under reduced pressure at 50-60°C.

Two drops of the ferric chloride solution were placed on two different portions of a No.1 Whatman filter paper. Two drops of the concentrated effluent were added to one of the spots containing $FeCl_3$. The paper was dried in the oven at 100°C for 3 minutes.

Similar procedures were repeated for the effluent collected from the Zeo Karb column.

<u>An approximate procedure for the quantitative estimation of amino acids</u>

The method used was that of Sorensen described by Mann & Saunders (1954).

1. About ten drops of phenolphthalein solution were added to 50 ml. of 40% formalin solution; 0.1 N NaOH was added very carefully from a burette until the solution was just faintly pink.

2. 2.0 gm of yeast extract dissolved in 100 ml. distilled water, pH was adjusted to 8 on addition of a few drops of N NaOH.

3. Ten millilitres of the yeast solution were transferred to a conical flask, two drops of phenolphthalein added; 0.1 N NaOH was added from the burette until the solution was just faintly pink.

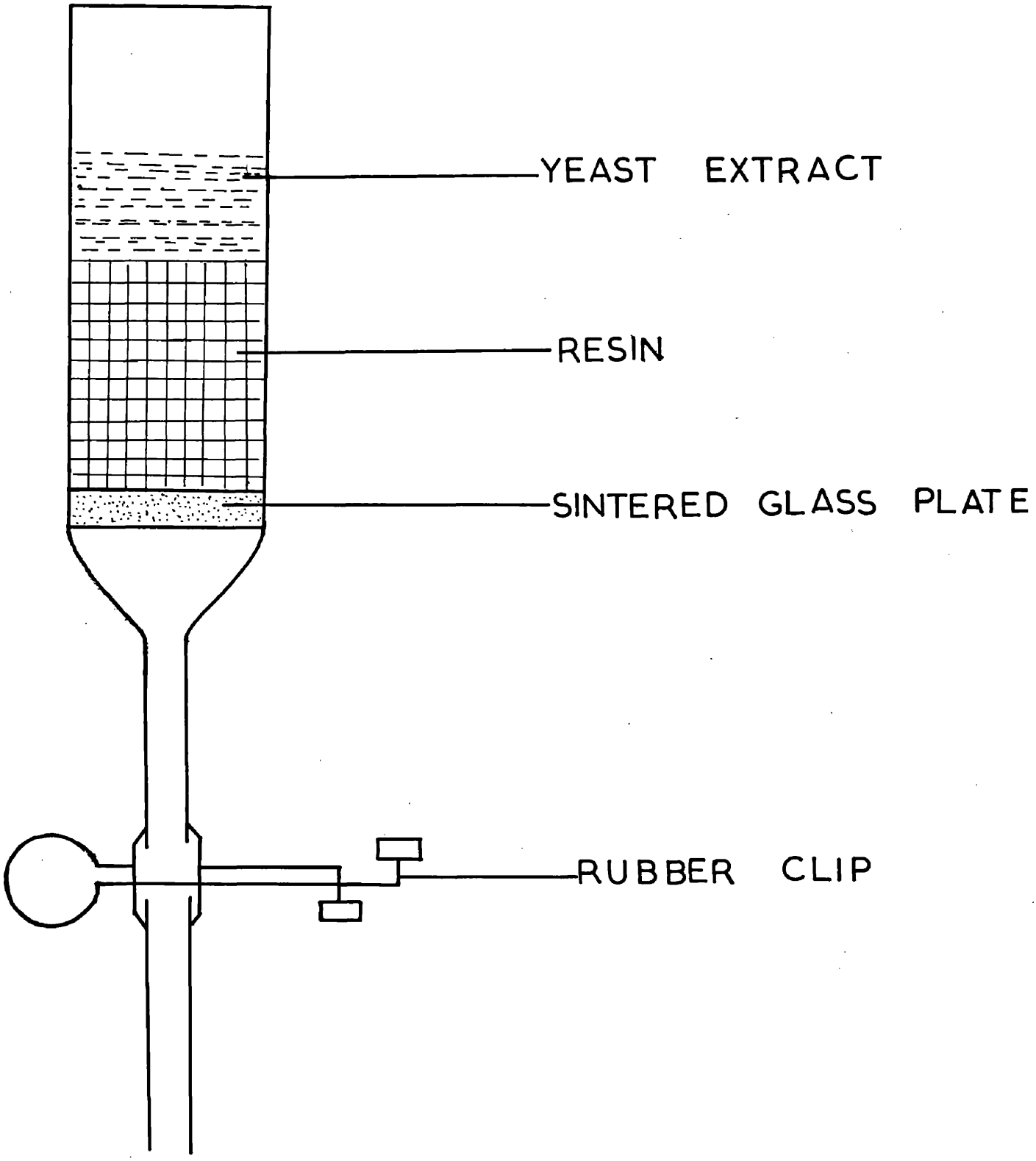
4. Five millilitres of the formalin solution in (1) were

added. The pink colour of the phenolphthalein disappeared and the solution became markedly acid. The solution was titrated against 0.1 N NaOH until the pink colour was just restored. The process was repeated with two further quantities of 2% yeast extract to obtain constant readings.

FIGURE 5

Diagram of an ion exchange resin column.

This consists of a glass tube about 16 cm long, the lower half of about 0.5 cm and the upper portion of about 1.0 cm internal diameter. The lower end is closed by means of a rubber tubing fitted with a clip, and a short glass tube is attached to the other end of the rubber tubing. Just above the lower half of the tube is fused a sintered glass plate to support the resin.


YEAST EXTRACT
RESIN
SINTERED GLASS PLATE
RUBBER CLIP

Results

(A) Development of D. phoxini in media

Firstly, the effects of mild alkaline hydrolysis of yeast was studied. Development in the basal + yeast medium was compared with that obtained in the basal + alkaline hydrolysed yeast medium and basal + A.A. + alkaline hydrolysed yeast medium respectively. Results are shown in Table 23.

There were fewer flukes producing sperms and vitellaria in the basal + hydrolysed yeast medium (culture 91B1-B3), than in the basal + yeast medium. But on addition of amino acids to the basal + hydrolysed yeast medium (cultures 91BA1-BA3) there was a considerable increase in the number of flukes producing sperms and vitellaria. In fact, development is just as good as that obtained in basal + yeast medium. This suggests that some of the alkaline labile amino acids in yeast extract are beneficial to the flukes.

The results obtained also indicate that yeast extract is not an essential source of B_{12}, biotin, riboflavin and pantothenic acid, all of which vitamins are destroyed in hot alkaline solution.

Table 23

Shows the effect of adding yeast extract, alkaline hydrolysed yeast extract and amino acids + alkaline hydrolysed yeast extract to basal medium.

Culture no.	Additive to basal medium (BSSG + HS + alb)	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
Williams et al. 1961	yeast extract								
	PERCENTAGE		90	65	56	44			
91B1	alkaline hydrolysed yeast extract	6	19	7	12	12	1	6	6.2
91B2		7	17	7	9	9	1	6	6.5
91B2		4	21	10	9	9	-	6	6.3
	PERCENTAGE	23	76	32	40	40	3		
91BA1	amino acids + alkaline hydrolysed yeast extract	1	24	15	16	14	1	6	6.2
91BA2		-	25	16	21	21	5	6	6.1
91BA3		-	25	13	19	16	-	6	6.2
	PERCENTAGE	1	99	59	75	68	8		

Acid hydrolysis of yeast

Table 24 shows the level of development attained in the basal + acid hydrolysed yeast with or without amino acids as compared with those in the basal + yeast with or without amino acids.

With the amino acid supplement in the medium, there appeared to be no significant difference between development obtained in cultures containing untreated yeast extract and those with acid hydrolysed yeast (cultures 56H1-H3). On the other hand, the number of flukes developing sperms is considerably less in cultures containing acid hydrolysed yeast without added amino acid. This difference could be attributed to the destruction of the amino acids which are heat labile in acidified yeast extract, suggesting that yeast was contributing some amino acids to the medium.

Table 24

Shows the effect of adding yeast extract and acid hydrolysed yeast extract to basal with and without amino acids.

Culture no.	Additive to basal medium (BSSG + HS + alb)	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
Williams et al. 1961	yeast extract (10 cultures)								
	PERCENTAGE		90	65	56	44			
Expt. 71 Table 29	acid hydrolysed yeast extract								
	PERCENTAGE	9	91	31	43	36			
Expt. 56 Table 25	amino acids + yeast extract								
	PERCENTAGE	8	85	75	69	69	5		
56H1	amino acids + acid hydrolysed yeast extract	-	25	22	17	16	1	6	6.3
56H2		1	24	16	14	13	-	6	6.3
56H3		2	17	10	11	10	-	7	6.7
	PERCENTAGE	4	88	64	58	52	1		

Dialysis of yeast

Table 25 compares development in the basal + dialysed yeast with or without amino acid and that in the basal + amino acid + whole yeast extract medium.

With the amino acid supplement in the medium, there appeared to be no difference in development in cultures containing dialysed yeast (cultures 56D1-D3, 60D1-D3) and those containing whole yeast extract (cultures 56C1-C3, 60C1-C3). On the other hand, the number of flukes developing sperms and vitellaria is considerably less in cultures containing dialysed yeast without added amino acid (cultures 90T1-T3).

Table 25

Shows the effect of adding yeast extract + amino acids (controls), dialysed yeast extract + amino acids and dialysed yeast extract to basal medium.

Culture no.	Additive to basal medium (BSSG + HS + alb)	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
56C1	amino acids + yeast extract	-	23	19	16	16	1	6	6.8
56C2		2	20	18	16	16	3	6	6.7
56C3		4	21	19	20	20	-	6	6.0
60C1		-	25	17	14	14	-	6	6.4
60C2		-	25	15	14	14	5	6	6.4
60C3		3	25	19	13	12	-	6	6.4
	PERCENTAGE	6	96	71	62	61	6		
*56D1	amino acids + dialysed yeast extract	3	19	13	11	11	-	6	6.8
*56D2		2	22	16	13	13	1	6	6.5
*56D3		4	21	11	14	13	1	6	6.3
+60D1		-	25	23	19	15	-	6	6.5
+60D2		1	24	18	15	13	-	6	6.6
+60D3		-	25	17	19	14	-	6	6.6
	PERCENTAGE	7	91	65	61	53	1		
90T1	dialysed yeast extract	15	10	6	5	3	-	6	6.4
90T2		19	6	3	4	4	-	6	6.2
90T3		14	11	3	3	2	-	6	6.2
	PERCENTAGE	64	36	16	16	12	0		

*Dialysed yeast prepared by Method I

Dialysed acid hydrolysed yeast

Wilson (1960) had shown that there was some activity in dialysed yeast; this was also confirmed from the results obtained in experiment 90 (Table 25; cultures 90T1-T3). To gain an idea of the nature of the non-dialysable nutritive factors of yeast, it was decided to study whether these factors were attached to proteins. This was done by subjecting the yeast to mild hydrolysis before dialysis, as mild hydrolysis would probably detach small molecular prothetic groups from proteins.

Table 26 shows development in the basal + acid hydrolysed yeast + amino acid medium compared with that in the basal + dialysed acid hydrolysed yeast with and without amino acid.

Development in cultures 60H1-H3 was poorer than in cultures 56H1-H3 (Tables 24 & 26), suggesting that some of the active fraction of yeast had been lost by dialysing the acid hydrolysed extract. This shows that part of the growth factor of dialysed yeast was probably a small molecular substance attached to a large one.

As was expected there was very little development in cultures containing basal + dialysed hydrolysed yeast (cultures 92H1-H3).

Table 26

Shows the effect of adding amino acids + acid hydrolysed yeast extract, amino acids + dialysed acid hydrolysed yeast extract and dialysed acid hydrolysed yeast extract to basal medium.

Culture no.	Additive to basal medium (BSSG + HS + alb)	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
Expt. 56 Table 24	A.A. + acid hydrolysed yeast extract								
	PERCENTAGE	4	88	64	58	52	1		
60H1	A.A. + dialysed acid hydrolysed yeast extract	2	23	16	14	14	-	6	6.2
60H2		5	20	9	8	7	-	6	6.5
60H3		4	18	8	8	6	-	6	6.5
	PERCENTAGE	15	81	44	40	36	0		
92H1	dialysed acid hydrolysed yeast extract	16	9	-	-	-	-	6	6.5
92H2		16	8	-	-	-	-	6	6.4
92H3		18	6	-	-	-	-	6	6.6
	PERCENTAGE	67	31	0	0	0	0		

Effluent of yeast extract through ion-exchange columns

To obtain a guide to the probable nature of the substances contributed by yeast to the medium, extracts were passed through ion-exchange columns.

Dowex I (Cl^-) at pH 8 was used which should absorb the acidic amino acids, and possibly some of the neutral amino acids as well, the acidic B vitamins, nucleic acids and bases (Samuelson, 1953). The activity of effluents of yeast extract passed through this column was tested in cultures.

Table 27 shows the development obtained when effluent of yeast from a Dowex I column was added to the basal medium with and without an amino acid supplement. The controls (cultures 20Y1-Y2) contained basal + amino acid + yeast extract.

The cultures were assessed on the development attained by the best 10 flukes in each culture. This method failed to indicate any differences in the level of development attained in each medium. However, this does not mean these media were all equal, only that they were all good enough to support a certain level of development. All that could be concluded was that anion exchange did not reduce the activity of the yeast by a major extent.

Table 27

Shows the effect of adding yeast extract + amino acids (controls), yeast effluent (Dowex I) + amino acids and yeast effluent (Dowex I) to basal medium

Culture no.	Additive to basal	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				
20Y1	amino acids + YE	10	10	8	10	10	10	10	1	10	51	5	7.1
20Y2		10	10	9	10	10	10	10	1	10	45	5	7.2
	PERCENTAGE	100	100	85	100	100	100	100	10				
20E1	amino acids + yeast effluent (Dowex I)	10	10	10	10	10	10	10	1	8	32	5	7.4
20E2		10	9	6	10	10	10	10	-	10	52	5	7.1
51A1		10	10	10	10	10	10	10	1	12	90	6	6.4
51A2		10	10	10	10	10	10	10	-	12	70	6	6.4
51A3		10	10	10	10	10	10	10	-	12	60	6	6.7
	PERCENTAGE	100	98	92	100	100	100	100	4				
51X1	yeast effluent (Dowex I)	10	10	8	10	10	10	8	-	12	72	6	6.7
51X2		10	10	10	10	10	10	9	-	12	84	6	6.8
51X3		10	10	9	10	10	10	9	-	12	66	6	6.8
	PERCENTAGE	100	100	90	100	100	100	87	0				

Because of the doubt that arose in interpreting the results of experiment 20/51 sampled by the best ten method it was decided to repeat this experiment and to include cultures using cation exchange effluent from acid hydrolysed yeast as well. The results (Table 28) showed that development in effluent Dowex I + amino acid (84D1-D3) was not as good as expected in yeast extract + amino acid (Table 30). The fall off in development, however, was far more apparent when the amino acid supplement was omitted, suggesting the importance of the acidic amino acids in yeast extract.

In basal supplemented by yeast effluent from a cation column, less than 5% of the flukes develop sperms and yolk reservoirs suggesting that most of the active fraction of yeast had been absorbed in the resin.

Table 28

A comparison in the level of development when yeast effluents from anion and cation exchange columns is added to basal ± amino acids.

Culture no.	Additive to basal medium (BSSG + HS + alb)	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
84D1	A.A. + yeast effluent (Dowex I)	1	24	9	15	13	-	6	6.4
84D2		2	23	9	15	13	2	6	6.2
84D3		-	25	11	17	16	2	6	6.3
	PERCENTAGE	4	96	39	63	56	5		
84C1	yeast effluent (Dowex I)	2	23	5	9	6	-	6	6.3
84C2		4	21	6	6	5	-	6	6.4
84C3		3	22	7	7	6	-	6	6.2
	PERCENTAGE	12	88	24	29	23	0		
84Z1	yeast effluent (Zeo Karb 215)	12	13	2	4	2	-	6	6.4
84Z2		14	10	1	1	1	-	6	6.4
84Z3		15	9	-	-	-	-	6	6.5
	PERCENTAGE	55	43	4	7	4	0		

As yeast extract was acid hydrolysed before it was passed through the cation exchange column, development in the basal medium supplemented with such effluent was compared with that obtained in the basal supplemented with acid hydrolysed yeast. Results are shown in Table 29.

There was a great loss in activity of the acid hydrolysed yeast when passed through the cation column, cf. cultures 71C1-C3 and 71Z1-Z3.

Table 29

Shows the effect of adding acid hydrolysed yeast extract and effluent of acid hydrolysed yeast extract (Zeo Karb 215) to basal medium.

Culture no.	Additive to basal medium (BSSG + HS + alb)	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
71C1	acid hydrolysed yeast extract	-	25	5	11	8	-	6	6.3
71C2		2	23	10	13	11	-	6	6.5
71C3		5	20	8	8	8	-	6	6.3
	PERCENTAGE	9	91	31	43	36	0		
71Z1	effluent of acid hydrolysed yeast extract (Zeo Karb 215)	20	5	1	0	0	-	6	6.2
71Z2		14	11	2	2	2	-	6	6.2
71Z3		19	6	1	0	0	-	6	6.2
	PERCENTAGE	71	29	5	3	3	0		

Table 30 gives a summary of the level of development reached by the flukes after cultivation for six days in the basal medium supplemented by various fractions of yeast extract + amino acids. Since very good cultures often had a few flukes in which development occurred, variations over 90% in the development of follicular testes are of little significance and are not given.

The figures in parentheses have been calculated to indicate the percentage loss in activity of the various yeast supplements in promoting maturation. For example, in medium 8a in which the yeast was acid hydrolysed before use, 31% of the flukes developed active sperms, compared with 65% in medium 8; the difference of 34 shows a loss in activity of 52%. Similarly development of genitalia in flukes in all other media (8a-f) derived from medium 8 is expressed as a percentage change.

In calculating the difference in media derived from medium 9, which differs from medium 8 series only in that an amino acid supplement was added, a correction factor has been used. This was necessary because some sperm and vitellaria are formed without yeast if amino acids are added (medium 2). Since the figure in parentheses show changes in the activity of the yeast, it was necessary to subtract the results of medium 2

from medium 9 to arrive at the proportion of development that could be attributed to the yeast. Thus, in medium 9a, active sperm occurred in 64% of the flukes compared with 70% in the untreated extract (medium 9). The loss in activity due to hydrolysis is therefore 6/60th (not 6/70th), since 10% of the flukes could be expected to form active sperm in the absence of any form of yeast (medium 2). Similarly there was a fall of 7 in the number of flukes producing vitellaria, which indicates a loss of 7/60th, i.e. 12%, in the ability of acid hydrolysed yeast to promote vitelline development.

Since considerable variation occurs in the same medium, little significance should be attributed to differences less than 20%.

Table 30

The level of development reached by D. phoxini after cultivation for 6 days at 40°C in a basal medium (BSSG + HS + alb) supplemented by various fractions of yeast extract (YE)

Medium no.	Supplement	Amino acids	No. of flukes cultures	Follicular testes %	Active sperm %	Vitellaria %	Yolk reservoir %
9	yeast extract	+	300/12*	90	70	65	56
8		-	250/10*	90	65	56	44
2	none	+	125/5*	68	10	5	3
1		-	150/6*	24	0	0	0
9a	acid hydrolysed YE	+	75/3 (Table 24)	90	64 (-10)	58 (-12)	52 (-8)
8a		-	75/3 (Table 29)	90	31 (-52)	43 (-23)	35 (-25)
9b	alkaline hydrolysed YE	+	75/3 (Table 23)	90	59 (-18)	75 (+17)	68 (+23)
8b		-	75/3 (Table 23)	76	32 (-51)	40 (-29)	40 (-9)
9c	dialysed YE	+	75/3 (Table 25)	90	75 (+8)	71 (+10)	56 (0)
8c		-	75/3 (Table 25)	36	16 (-75)	16 (-71)	12 (-73)
9d	dialysed fraction of acid hydrolysed YE	+	75/3 (Table 26)	81	44 (-43)	40 (-42)	36 (-38)
8d		-	75/3 (Table 26)	31	0 (-100)	0 (-100)	0 (-100)
9e	effluent from anion exchange pH 8	+	75/3 (Table 28)	90	39 (-52)	63 (-3)	56 (-0)
8e		-	75/3 (Table 28)	90	24 (-63)	29 (-48)	23 (-48)
8f	effluent from cation exchange	-	150/6 (Tables 28 & 29)	36	5 (-92)	5 (-91)	3 (-91)

*Williams et al. (1961)

The figures in parenthesis shows % increase or decrease reached by flukes in the medium compared with that in basal + yeast extract ± amino acids

(B) Estimation of B_6 and amino acids in yeast effluents from anion and cation exchange columns

Table 31 shows the results of a spot test for the detection of vitamin B_6 (see Methods) in yeast effluents from anion and cation exchange columns.

Table 31

Test made	Colouration	Inference
$FeCl_3$ spot	yellow	—
$FeCl_3$ spot + effluent (anion exchange)	brownish-red	B_6 present
$FeCl_3$ spot + effluent (cation exchange)	yellow	B_6 absent

Formaldehyde titrations (see Methods) were carried out on 2% yeast extract and on effluents of 2% extract passed through anion and cation exchange columns respectively (Table 32).

Table 32

Solution	Volume of 0.1 N NaOH required for neutralisation	% loss of amino acid
2% yeast extract	5.5 - 5.7 ml.	0
effluent (anion exchange)	4.4	20 - 23
effluent (cation exchange)	0.45	93 - 96

It must be emphasised that the results obtained are only roughly quantitative; due to the colour of the yeast extract it was difficult to obtain very accurate end-points in titrations using phenolphthalein as indicator.

Discussion

The value of yeast extract has been assessed in a medium containing albumen and horse serum. There may be many substances essential for the growth of Diplostomum phoxini which occur in adequate quantities in serum (see Section VI), and therefore need not be included in a supplement designed to replace yeast extract in the medium. To gain an idea of the properties and size of the molecules of the growth promoting factors of yeast, a series of analytical procedures were applied to it and the effects of the activity of the extract measured.

Mild acid hydrolysis had no effects when amino acid supplement was added (Tables 24 & 30) which suggested that the B vitamin components which are destroyed by boiling in N HCl, viz. pantothenic acid (Lepkovsky, 1954), B_{12} (Lester-Smith, 1956) and folic acid (Daniel & Kline, 1947) are not required from yeast extract; substantiating similar results obtained in Section I. On the other hand, in the absence of the amino acids development was retarded (Table 30); it would seem that the flukes required a source of at least one of the acid labile amino acids, serine, threonine and tryptophane, all of which occur in yeast extract, though serine only in trace quantities (Difco, 1958; Pyke, 1956).

Similar results to those obtained with acid hydrolysed yeast extract were obtained using alkaline hydrolysed extract. With the amino acid supplement the development attained was not significantly different from that in untreated yeast extract (Table 30). Alkaline hydrolysis destroyed vitamin B_{12} (Hartley et al., 1950), pantothenic acid (Robinson, 1951), thiamin (Rosenberg, 1945), riboflavin (Wagner-Jauregg, 1954) and biotin (Merck, 1960). The destruction of vitamin B_{12} and pantothenic acid substantiates conclusion reached using acid hydrolysed yeast, it also confirms results obtained in Section I that vitamin B_{12}, pantothenic acid, riboflavin, biotin and thiamin in B vitamin solution I were not contributing any nutritive factor to the medium. Without the amino acids sperm production was reduced by about 50% and vitellaria by 30% (Table 30). Alkaline hydrolysis destroys cysteine, cystine, and arginine, in addition to the acid labile amino acids, serine and threonine. The reduction in development in the absence of an amino acid supplement suggests that some of the amino acids in yeast are essential for the flukes as was deduced in Section I.

The results obtained with dialysed yeast indicated that in the presence of an amino acid supplement, the importance of yeast extract was in the large molecular fraction (Tables 25 & 30). In the absence of amino acids,

dialysed yeast was of little value. It appeared, therefore, that yeast was contributing (I) a dialysable fraction, the active part of which consisted of amino acids and (II) a large non-dialysable fraction. No attempt was made to determine which amino acids in yeast the worms were utilising as this aspect of nutrition will be more easily studied when a successful medium containing defined substances is developed.

To test the possibility that the active part of the non-dialysable fraction was a small molecule attached to a protein, and not a large molecule, yeast extract was first hydrolysed and then dialysed. Whereas separately neither of these procedures caused loss of activity (Tables 24 & 25), when combined this resulted in a loss of half the activity of the yeast extract (Table 30). This suggests that at least part of the activity in the non-dialysable fraction was due to a small molecular substance or substances which are freed by acid hydrolysis without damage, but therefore lost on dialysis.

To obtain a guide to the probable nature of the substances contributed by yeast, extracts were passed through ion exchange columns. An acid hydrolysed extract was passed through a cation exchange column, Zeo Karb 215; the resin used, absorbs in the H^+ form

at pH 4 all the amino acids, even aspartic and glutamic (Samuelson, 1953). A rough estimate of the loss of amino acids after passage through the column, showed that 93-96% of the amino acids were left in the resin (Table 32). This resin also absorbs proteins, bases, and B vitamins. No trace of vitamin B_6 was detected in the effluent (Table 31). Not surprisingly, therefore, the effluent showed almost complete loss of activity (Tables 29 & 30).

The results obtained using the effluent from anion exchange column, Dowex I (Cl^-) are more difficult to interpret. There was only about 20-23% loss of amino acids in the effluent (Table 27). This loss is probably due to the adsorption of the acidic amino acids. All that can be concluded is that since vitellaria production remained normal in the medium with amino acid supplement (Table 30), the non-dialysable factor passed through the column and hence is basically charged. The effluent was found to give a positive reaction for the presence of vitamin B_6 (Table 31). The poor development obtained when the amino acid supplement was omitted is probably due to the total loss of the acidic amino acids which might have been held loosely by the resin. The effluent used did not contain a washing from the column.

It could then be concluded that the active

non-dialysable component of yeast extract was a small molecular substance, resistant to mild acid and alkaline hydrolysis, basically charged, and usually occurring in a large non-dialysable complex. Of the substances reported in yeast, pyridoxine or some other form of vitamin B_6 fitted this description. This substance occurs as a non-dailysable protein complex in yeast (Kuhn & Wendt, 1938) is basically charged and resistant to 5N acid and alkali at 100°C (Robinson, 1951). It must be added, however, that the proportion of B_6 that remains in a non-dialysable complex after autolysis which occurs in the preparation of yeast extract is not known. Certainly, proteins are not completely hydrolysed as is shown by formol titration relative to total nitrogen (Sykes, 1956). In Section I, it was shown that pyridoxine gave only about 70% of the development of yeast in the medium (Table 13), and that, only if the pyridoxine concentration was about 500x that in yeast extract. This might suggest that the vitamin B_6 in yeast was in a more active form, or that yeast was contributing some other factors of which the effects are partly replaced by a high concentration of pyridoxine.

Summary

1. Yeast is contributing (i) a dialysable fraction and (ii) a large non-dialysable fraction to the medium.

2. The active part of the dialysable fraction consisted of amino acids.

3. The non-dialysable component of yeast extract is a small molecular substance, resistant to mild acid and alkaline hydrolysis, basically charged and usually occurring attached to a large molecular complex.

4. It is suggested that the activity of the non-dialysable component of yeast is in part due to pyridoxine or some other form of vitamin B_6.

SECTION V: THE REPLACEMENT OF HORSE SERUM IN CULTURE MEDIA

Introduction

In microbiology, it is almost axiomatic that a serum requirement indicates a need for lipids (Hanks, 1955). It was thought that serum was probably supplying some lipoid nutrients for the flukes. This view was strengthened by the fact that hen egg yolk, which is rich in lipids, is an excellent medium for D. phoxini (Bell, 1958; Wilson, 1960). Wilson also demonstrated that the activity of serum was confined to its non-dialysable fraction; this fraction included not only the proteins or the small molecules bound to or incorporated in the proteins but also the lipids in serum.

Attempts were made to replace serum in the medium in two ways. Firstly, by preparing supplements of lipoid material, viz. egg-lecithin + cholesterol and a solution of fat-soluble vitamins A,D and E. Secondly, by the selection of a natural product which would wholly replace horse serum. It was thought that if a water soluble extract could be found, this would be an advantage, as experience with the yeast extract had shown that a water extract could easily be fractionated. Two substances were tested for activity, raw liver extract and

chick embryo extract. Dougherty et al. (1951 et seq.), demonstrated that blood plasma, chick embryo extract and raw liver extract were interchangeable, as constituents of a medium for the cultivation of free living nematodes.

As various components of bovine serum (e.g. bovine albumin, globulins) were easily available commercially, whereas purified fractions from horse serum were not, experiments were carried out to discover whether bovine serum was as good as horse serum.

In most of the experiments described in this section, autoclaved liver extract was used instead of yeast extract, i.e. control medium consisted of BSSG + albumen + serum + ALE (autoclaved liver extract). As was shown in Section II, ALE has an equal growth promoting effect as yeast extract when added to the other substances in the control medium.

Materials and Methods

(1) Egg-lecithin and cholesterol solutions

Hen-egg-lecithin was used as a source of lipids. A solution was prepared as described in Section III. A cholesterol solution was prepared by a similar method as that used for the lecithin solution. 30 mg cholesterol dissolved in 3 ml.

alcohol was diluted with 5 ml. aqueous Tween 80 (5% Tween 80 in water) and 92 ml. sterile distilled water. 0.3 ml. of cholesterol solution and 0.3 ml. of lecithin solution were added to a medium consisting of BSSG (8 ml.) autoclaved liver extract (1 ml.) and albumen (1.5 ml.).

(2) Fat soluble vitamins solution

a. Ten drops of Adexolin liquid were dissolved in 3 ml. alcohol in a universal container and left standing overnight in an ovem at 80°C. (Adexolin liquid is a concentrated solution of vitamins A and D in oil supplied by Glaxo Laboratories. Each drop containing 500 international units of vitamin A and 80 units of vitamin D.)

b. Fifty milligrams d-alpha-tocopheryl acid succinate were dissolved in 3 ml. absolute alcohol in a universal container and left standing overnight. Both solutions from (a) and (b) were transferred into a 125 ml. bottle shaken for a few seconds, 5 ml. of aqueous Tween 80 and 89 ml. sterile distilled water added. The bottle was closed and shaken in a mechanical shaker for 5 minutes. 1 ml. of the solution was added to a medium consisting of 6 ml. BSSG, 1 ml. autoclaved liver

extract (ALE), 1.5 ml. pyridoxine hydrochloride, 0.5 ml. thiamin hydrochloride (40 mg. thiamin in 50 ml. water), 1.5 ml. albumen and 1.5 ml. amino acid.

(3) Bovine Serum

Bovine serum was obtained in a sterile form in 10 ml. ampoules from Difco Laboratories. This was added to a medium containing BSSG, albumen and yeast extract to constitute 25-30% of the medium and the pH adjusted to 6.9.

(4) Raw liver extract

Several liver extract were prepared from liver from three different species using slight modifications of the methods described in Section II.

a. Raw liver extract (RLE pigeon)

Prepared as described in Section II.

Three millilitres of RLE (pigeon) were added to a medium containing 1 ml. autoclaved liver extract (ALE), 6 ml. glucose balanced salt solution and 1.5 ml. albumen.

b. RLE (rat)

The method used for the preparation of raw liver extract (rat) was a slight modification of that used in (a).

Ten grams of rat liver were homogenised in 40 ml. glucose balanced salt solution, centrifuged at about 3,000 g for 12 minutes, during which time the temperature rose to 21°C. The supernatant was pipetted into a sterile 125 ml. bottle, 4,000 units of penicillin and 2.5 mg of streptomycin added, and left overnight at 4°C. This was seitz filtered and stored at 4°C.

Two millilitres of RLE (rat) were added to a medium containing 1.0 ml. yeast extract, 3.5 ml. BSSG, 1.5 ml. amino acid and 1.0 ml. albumen. The pH was adjusted to 6.9.

c. RLE (horse)

To ensure that the liver extract would not lose activity due to a rise in temperature during the centrifugation (as in (b)), a refrigerated centrifuge was used. Horse liver was used because horse serum has been shown to be active in cultures (Wyllie et al., 1960). The method used is a modification of that described by Nicholas, 1959.

Fresh liver from a horse obtained from the slaughter house within 10 minutes of the death of the horse was conveyed to the laboratory in an ice box. 120 gm of the liver was homogenised in

120 ml. M/15 KH_2PO_4. The homogenate was spun in a refrigerated centrifuge at 4°C at 5,000 g for 30 minutes. The supernatant was passed with difficulty through a seitz filter. About 40 ml. extract was obtained. This was divided into two lots of 20 ml. each. One lot was passed through a second seitz filter, and the second lot sterilised by filtering through sintered glass. The former product was septic and was not incorporated in culture medium.

Two millilitres of the sterile extract were added to a medium containing autoclaved liver extract (1 ml.), BSSG (2 ml.), albumen (1 ml.), 1,000 units of penicillin and 0.5 mg. of streptomycin.

(5) Chick embryo extract (EE_{50})

The method used was that described by Paul (1959) using 11-day-old chick embryos. The following procedures were applied.

a. The shells of eight incubated eggs were sterilised with alcohol which was left to dry in the sterile cabinet.

b. The blunt end of each was cut opened with a pair

of sterile pointed scissors.

c. The shell over the air space was removed to expose the membrane.

d. The membrane was loosened and removed with a pair of sterile forceps.

e. A curved pair of forceps was slipped under the embryo's neck, which was extracted gently from the egg and deposited in a dish containing sterile BSS.

f. When all the embryos had been removed, each was rinsed three times in the salt solution to remove all the blood and yolk.

g. The embryos were then dropped into the barrel of a 30 ml. sterile syringe, two at a time, and the plunger inserted gently.

h. The tip of the syringe was inserted into a graduated centrifuge tube and the embryo mash expressed.

i. An equal volume of BSS was added to the pulp, stirred with a sterile glass rod and left for 30-90 minutes at room temperature and then centrifuged for 20 minutes at 2,000 g.

j. The supernatant (EE_{50}) was distributed in 10 ml. aliquots in universal containers. A sterility test was made by incubating one of the containers at 39°C for 48 hours. Any cloudiness in the extract indicated presence of bacteria.

k. The extract was stored at -15°C in the dark. Before use it was thawed slowly and the precipitate formed was dispersed evenly in the solution before pipetting the preparation into culture media.

Embryo extract was tested at two levels: 15% and 25% in the final medium.

(6a) <u>Embryo extract at 25% level in the medium</u>

The medium containing embryo extract at a level of 25% was prepared as follows:

25 ml. embryo extract (EE_{50})
5 ml. 4% yeast extract
7.5 ml. amino acids
12.5 ml. BSSG

were added together in a 125 ml. sterile bottle and dispersed in 10 ml. aliquots in universal containers; 1.5 ml. albumen added to each and the pH adjusted to 6.9.

(6b) <u>Embryo extract at 15% level in the medium</u>

Three millilitres embryo extract (EE_{50}) were added

to 1 ml. yeast extract (4%) 1.5 ml. amino acids, 4.5 ml. BSSG, 1.5 ml. albumen, 1,000 units penicillin and 1 mg. streptomycin. The pH was adjusted to 6.9.

(7) Heated embryo extract (EE_{50})

Twelve millilitres of freshly prepared embryo extract (EE_{50}) were heated in a water bath at 80°C for 10 minutes. This was then pipetted into the medium to give a level of 15% as described in 6b.

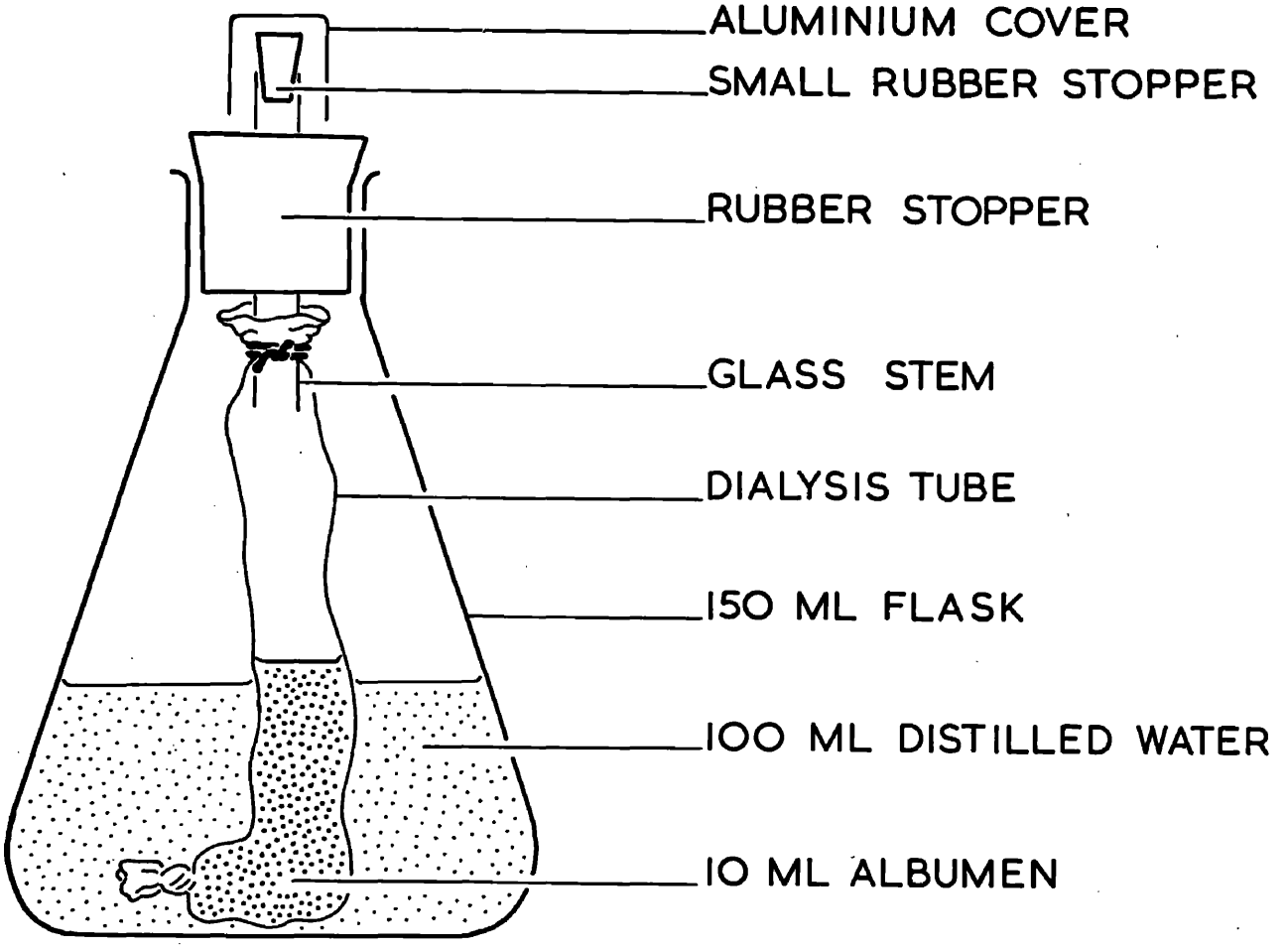
(8) Dialysed embryo extract

The method used was a modification of that described by Harris (1952). Freshly prepared embryo extract (EE_{50}) was dialysed in a 150 ml. conical flask. Each unit consisted of a short stem of glass tubing which passed through a rubber stopper in the mouth of the flask, and to which was affixed by a string an appropriate length of dialysing tubing (½-inch diameter). A knot was made on the other end of the tube. The dialysing bag was filled with distilled water and the entire unit (figure 6) sterilised by autoclaving at 15 lbs/sq.in. for 15 minutes. The water was emptied from the dialysing bag and about 7-8 ml. of sterile embryo

extract introduced by a pipette into the bag and the glass tube closed with rubber stopper. The unit was then transferred into another flask containing BSS at 4°C. The flask was housed in a sterile beaker covered with a crystallising dish and stored at 4°C. The dialysing fluid (BSS) was changed daily for four days. Aseptic precautions were observed throughout. The dialysed extract was removed with a sterile pipette and stored at 4°C in universal containers. Only freshly prepared dialysed embryo extract was used for cultivation. As some precipitation occurred during dialysis; the precipitate formed was evenly dispersed in the solution by shaking before pipetting the preparation into culture media. 3.0 ml. of dialysed embryo extract were added to each culture tube consisting of 1.5 ml. amino acids, 1.0 ml. of 4% yeast extract, 1.5 ml. albumen, 3.5 ml. BSSG, 1,000 units penicillin and 1 mg. streptomycin. The pH was adjusted to 6.9.

FIGURE 6

Diagram of the apparatus used to prepare (a) dialysed embryo extract, (b) yeast dialysate.


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

Results

The basal medium used in cultures described in this section contained albumen and glucose balanced salt solution.

Firstly, an experiment was set up to demonstrate the importance of horse serum in a medium and to compare its effects with a solution of egg-lecithin + cholesterol (Table 33).

In basal + autoclaved liver extract (ALE) only a few flukes formed early testes; the rest had long thin posterior lobes with no distinct genital rudiments (cultures 48W2-W3). The addition of egg-lecithin + cholesterol to this medium did not improve the results (cultures 48L1-L3).

In the controls, basal + ALE + horse serum, there was a reasonable development of male genital structures in both cultures and good production of vitellaria in culture 48C2. Other experiments using ALE (e.g. experiment 61, Table 34) suggests culture 48C1 abnormal rather than culture 48C2.

Table 33

Shows the effect of adding (a) lecithin + cholesterol, (b) horse serum to basal + autoclaved liver extract (ALE).

Culture no.	Additive to basal + ALE medium	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubated	Fina pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
48W2	none	4	-	-	-	-	-	-	-	8	30	6	6.4
48W3		2	-	-	-	-	-	-	-	8	62	6	6.0
	PERCENTAGE	30	0	0	0	0	0	0	0				
48L1	lecithin + cholesterol	6	-	-	-	-	-	-	-	10	60	6	6.5
48L2		7	-	-	-	-	-	-	-	10	50	6	6.8
48L3		2	-	-	-	-	-	-	-	8	72	6	6.7
	PERCENTAGE	50	0	0	0	0	0	0	0				
48C1	horse serum	10	8	3	10	1	3	1	-	10	65	6	6.3
48C2		10	7	5	10	10	10	9	-	12	75	6	6.5
	PERCENTAGE	100	75	40	100	55	65	50	0				

In Table 34 are shown the results of an experiment designed to test whether the fat-soluble vitamins A, D and E can replace the effect of serum in a medium.

In basal + ALE + A.A. about 60% of the flukes developed follicular testes, but only about 20% formed active sperms and 10% formed vitellaria and yolk reservoirs.

The addition of the fat-soluble vitamins solution to the above medium (cultures 61A1-A3) did not improve development. However, on supplementing the basal + ALE medium with horse serum, over 50% of the flukes produced active sperms, vitellaria and a yolk reservoir. These results suggest that the fat-soluble vitamins alone are an unsuitable substitute for serum in cultures.

Table 34

Shows the effect of adding (a) vitamins A, D and E, (b) horse serum to basal + amino acids + autoclaved liver extract (ALE).

Culture no.	Additive to basal + amino acid + ALE	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
61X2	none	7	17	6	6	5	-	6	6.1
61X3		10	13	5	1	-	-	6	6.1
	PERCENTAGE	34	60	22	14	10	0		
61A1	vitamins A, D & E	8	9	5	-	0	-	6	6.3
61A2		15	3	2	1	0	-	6	6.2
61A3		11	11	4	1	0	-	6	6.1
	PERCENTAGE	45	31	15	3	0	0		
61C1	horse serum	-	25	15	13	11	-	6	6.5
61C2		3	22	14	14	13	-	6	6.8
61C3		2	23	13	15	14	-	6	6.7
	PERCENTAGE	7	93	56	56	51	0		

The growth promoting powers of bovine and horse serum in cultures are compared in Table 35.

When the basal + yeast medium was supplemented with bovine serum, only about 10% of the flukes developed sperms and vitellaria (cultures 101B1-B3, 102B1-B3). These results were considerably poorer than that obtained when the basal + yeast medium was supplemented with horse serum (cultures 102C1-C2) suggesting that bovine serum was not a suitable replacement for horse serum in the medium.

Table 35

Development in basal + yeast supplemented by (a) bovine serum and (b) horse serum.

Culture no.	Additive to basal + yeast extract	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
101B1	bovine serum	5	20	2	3	1	-	6	6.1
101B2		5	20	5	6	6	-	6	6.1
101B3		7	18	2	1	1	-	6	6.0
102B1		5	20	2	1	1	-	6	6.3
102B2		3	22	5	3	2	-	6	6.2
102B3		4	21	-	-	-	-	6	6.3
	PERCENTAGE	19	81	11	9	7	0		
102C1	horse serum	-	25	11	7	7	-	6	6.4
102C2		1	24	10	11	10	-	6	6.4
	PERCENTAGE	2	98	42	36	36	0		

In Table 36 are shown the results obtained with water extracts of raw liver from pigeon and the rat respectively, when each was tested as a replacement of horse serum in a medium.

When RLE (pigeon) was added to the basal + ALE medium, only 5% of the flukes developed active sperms as compared with 56% in control cultures containing horse serum (cf. cultures 62X1-X3 and 62C1-C3).

RLE (rat) seemed to produce some toxic effects in the medium as the flukes were all dead on the fourth day. A similar result was obtained using RLE (horse). (This is not recorded in Table 36.) No bacterial infection was detected on examination of smears of the media stained with methylene blue, but it was felt that a more sensitive test for identifying bacteria might probably have revealed that RLE (rat and horse) had some bacterial infection which caused the early death of the flukes.

Table 36

Shows the effect of adding horse serum (controls) and raw liver extracts to basal (BSSG + alb) + yeast or autoclaved liver extract.

Culture no.	Additive to basal (BSSG + alb)	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
100R1	yeast extract + horse serum	1	24	12	11	9	1	6	6.4
100R2		3	22	11	12	11	-	6	6.4
100R3		-	25	9	13	12	2	6	6.4
	PERCENTAGE	5	95	43	49	43	4		
61C1	ALE + horse serum	1	24	15	11	5	-	6	6.6
61C2		2	23	11	15	14	-	6	6.1
61C3		-	25	16	15	13	-	6	6.4
	PERCENTAGE	4	96	56	55	43	0		
62X1	ALE + RLE (Pigeon)	11	7	1	-	-	-	6	6.1
62X2		13	12	2	-	-	-	6	6.2
62X3		14	4	1	-	-	-	6	6.2
	PERCENTAGE	51	31	5	0	0	0		
100N1	yeast extract + RLE (rat)	FLUKES AT METACERCARIAL STAGE COVERED WITH GLOBULES						2	6.4
100N2		FLUKES ALL DEAD						4	6.4
100N3		" " "						4	6.4

Table 37 compares the growth promoting properties of embryo extract and horse serum when each was added to a medium consisting of basal + amino acid + yeast. Embryo extract was tested at 15% and 25% levels in the final medium respectively. The table also shows the effects of dialysis and heat on the growth factors of embryo extract.

There seemed to be very little difference in development when the basal + A.A. + yeast was supplemented either with embryo extract at 15% level or by horse serum at 25-30% level; compare cultures 96E1-E3 and 94C1-C2, 96C1-C2, 101C1-C2, suggesting that embryo extract was a suitable replacement for serum in the medium. At 25% level (cultures 94N1-N3) results were a little poorer, particularly obvious in the low sperm production, this may have been chance variation or be indicative that too high a concentration of embryo extract had been added.

There was only a little loss of activity when the embryo extract was dialysed before used as a supplement, cf. cultures 101D1-D3 with 96E1-E3, suggesting that the growth factors in embryo extract were large molecules or small molecules incorporated or bound with large ones.

Heating the extract to 80°C for 10 minutes resulted in a complete loss of its activity, suggesting that the nutritive factors are thermolabile. Compare cultures 101H1-H3 and 96E1-E3.

Table 37

Shows the effect of adding horse serum (controls) and various samples of embryo extract to basal + amino acids + yeast medium.

Culture no.	Additive to basal + A.A. + yeast	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
94C1	horse serum	3	22	17	15	14	-	6	6.3
94C2		1	24	21	14	13	-	6	6.3
96C1		-	25	12	10	10	-	6	6.3
96C2		2	23	12	11	9	-	6	6.1
101C1		-	25	19	14	14	1	6	6.4
101C2		-	25	16	14	8	-	6	6.4
	PERCENTAGE	4	96	65	52	45	1		
94N1	embryo extract at 25% level	-	25	6	11	7	-	6	6.3
94N2		-	25	10	14	12	-	6	6.4
94N3		-	25	9	11	10	-	6	6.4
	PERCENTAGE	0	100	33	48	39	0		
96E1	embryo extract at 15% level	-	25	12	12	11	1	6	6.1
96E2		-	25	14	17	14	1	6	6.1
96E3		-	25	13	13	11	3	6	6.1
	PERCENTAGE	0	100	52	56	48	7		
101D1	dialysed embryo extract at 15% level	5	20	12	12	9	-	6	6.2
101D2			25	10	7	6	-	6	6.4
101D3		8	17	11	11	8	-	6	6.3
	PERCENTAGE	17	83	44	40	31	0		
101H1	embryo extract heated to 80°C for 10 minutes	10	15	2	-	-	-	6	6.4
101H2		12	13	1	-	-	-	6	6.3
101H3		12	13	-	-	-	-	6	6.2
	PERCENTAGE	45	55	4	0	0	0		

Discussion

The addition of horse serum to a medium containing basal + ALE with or without amino acids greatly increases the growth promoting properties of the medium (Tables 33, 34 & 35). This effect of serum in the medium was neither replaced by egg-lecithin + cholesterol (Table 33) nor by the fat-soluble vitamins A, D and E (Table 34) suggesting that the essential factors in serum are probably not confined to the lipids or the fat-soluble substances.

The sample of bovine serum used did not give the same effects as horse serum in the medium (Table 35). Wilson (1960) also found that a sample of calf serum was inactive in cultures. Although no information was obtained about the preparation of the bovine serum, the fact that the horse serum used in cultures was always prepared from recalcified plasma might be of some significance.

No definite conclusions could be drawn from the experiments designed to replace serum by raw liver extract (RLE) in the medium; whether the poor results were due to toxic components in the extract (cf. Silverman, 1959) or whether the media were infected cannot be decided with certainty. The liver extract was doubly seitz filtered but being a very miscous substance, high

pressure was needed to force it through the pad - a procedure known to be unreliable in that bacteria may also be forced through the pad. The first step in any future work using raw liver extract must be the development of a better method of sterilisation.

Aqueous extract of 11-day-old chick embryos (EE_{50}) added to the basal + yeast + amino acid medium instead of horse serum to constitute a level of 15% embryo extract in the final medium gave satisfactory results (Table 37) suggesting that the nutritive substances of horse serum are contained in 11-day embryo extract. At 25% level, the results were a little poorer, particularly obvious in the low sperm production, this may have been chance variation or be indicative that too high a concentration of embryo extract had been added. All the experiments with embryo extract with the exception of experiment 101 were carried out with extracts which had been stored at -15°C for a few days; although the extract was clear when prepared initially, a precipitate was formed after freezing and thawing. This precipitate was uniformly dispersed by shaking before pipetting the preparation into culture media. It is possible, therefore, that the flukes take the embryo extract in the form of fine particles.

Due to the labile nature of embryo extract, only one successful attempt was made at fractionation. Only a

little loss of activity was observed when the extract was dialysed at 4°C for 90 hours, showing that the nutritive factors of chick embryo extract were in its non-dialysable fraction (Table 37). This result is interesting as Wilson (1960), found, in the presence of yeast and albumen in the medium, the nutritive properties of serum was confined to its non-dialysable fraction. This fraction in both material (horse serum and embryo extract) included the proteins, lipids and certain small molecules bound or incorporated in the proteins.

Heating the embryo extract at 80°C for 10 minutes resulted in a complete loss of its activity (Table 37; cultures 101H1-H3). Two possible explanations which could account for this loss of activity of the extract are:

(i) on heating the extract, some coagulation occurred and the extract was converted to a form in which the flukes could not use it;

(ii) the nutritive factors of embryo extract are thermolabile.

The latter explanation seems more likely as Hopkins (1960) observed a similar temperature effect in the cultivation of D. phoxini in a diphase medium containing

yolk as the solid phase and BSSG as the liquid phase. He found that good development was obtained in solid plates which were made by heating yolk at temperatures not exceeding 70°C; plates made at 80°C and at higher temperatures gave very poor development.

Embryo extract earned an early and unfortunate reputation as a useless fluid in cultures in which adequate concentrations of serum were present (Bell & Hopkins, 1956; Section III, this thesis). It is, however, a valuable substitute for serum in the medium. Dougherty et al. (1953) found that embryo extract or human blood plasma were suitable replacements for raw liver extract in a medium for the in vitro cultivation of the free-living nematode, Rhabditis briggsae. The active fraction of the raw liver extract was found to be non-dialysable and was destroyed on heating. It is therefore possible that the flukes and the free-living nematodes have some common nutritional requirements.

Since the replacement of horse serum by embryo extract did not produce better development, and as a considerable amount was already known about the effect of different fractions of serum, it was decided to postpone further investigations of the embryo extract and to apply further analytical procedures to horse serum.

Summary

1. The addition of horse serum to a medium containing BSSG + albumen + autoclaved liver extract with and without amino acids increases the growth properties of the medium.

2. Egg-lecithin + cholesterol or vitamin A, D and E are unsuitable replacements for serum in the medium.

3. Raw liver extract as a replacement for serum in the medium is an ineffective supplement.

4. Eleven-day chick embryo extract supplies the nutritive factors of serum when added to a medium containing BSSG + albumen + yeast + amino acids.

5. The growth factors of embryo extract are thermolabile and non-dialysable.

SECTION VI: HORSE SERUM AS A NUTRIENT IN CULTURE MEDIA

Introduction

Horse serum shows considerable growth effects when added to a medium containing BSSG + yeast extract + albumen with and without amino acids (Wilson, 1960). It was already suspected that in the presence of yeast and albumen, the important component that serum was contributing was in its non-dialysable fraction. Wilson (1960) in her preliminary experiments came to the conclusion that the gamma-globulin fraction in particular was important. In order to discover the important protein fractions of dialysed serum, development was therefore studied in a medium containing BSSG + yeast extract + albumen + amino acids supplemented by various fractions of equine serum proteins.

Since the main object of this work was to obtain a purely synthetic medium for the development of the flukes, it was decided to replace yeast in the medium by B vitamins and amino acids as these have been shown to be the most active components of yeast in the medium (Sections I & IV), and to test whether or not good development could be obtained by supplementing the medium with dialysed serum. As a result of the work,

Table 38

Bacto-yeast extract.

	Per cent
Ash	10.1
Total N	9.18
Chloride	0.190
Sulfur (total)	1.39
	PPM
Arsenic	0.11
Manganese	7.8
	Per cent
Phosphorus	0.89
Iron	0.028
SiO_2	0.052
Potassium	0.042
Sodium	0.32
Magnesium	0.03
Calcium	0.0406
Arginine	0.78
Aspartic acid	5.1
Glutamic acid	6.5
Glycine	2.4
Histidine	0.94
Isoleucine	2.9
Leucine	3.6
Lysine	4.0
Methionine	0.79
Phenylalanine	2.2
Threonine	3.4
Tryptophane	0.88
Tyrosine	0.60
Valine	3.4
	µg per gram
Pyridoxine	20.0
Biotin	1.4
Thiamine	3.2
Nicotinic acid	279.0
Riboflavin	19.0

it was found that the small molecular components of serum were important in the absence of yeast, and investigations were carried out to discover the active components.

Materials and Methods

Normal horse serum (No. 2) was obtained from Burroughs Wellcome.

Horse serum protein fractions

Horse serum protein fractions, viz, albumin (fraction V), alpha-globulins (fraction IV), beta-globulins (fraction III) and gamma-globulins (fraction II) were obtained from Light & Co. Solutions of globulin fractions were prepared by dissolving 1 gm of the required fraction in 30 ml. balanced salt solution and sterilising by seitz filtration.

A similar method was used for the albumin but using water as the solvent.

One millilitre of each solution was used in each culture.

Dialysis of horse serum

In early experiments and in experiment 114, 150 ml. of horse serum was dialysed for 24 hours against several changes of distilled water; in later experiments the

serum was dialysed against running water at 15-17°C for 72 hours. On completion of dialysis, the volume was measured, usually it was found to have increased by one half. The volume was then increased further to 375 ml. by the addition of a salt solution which contain the appropriate amounts of salt for 375 ml. BSSG, and seitz filtered.

It was used freshly prepared or after storage at -15°C up to one week.

Dialysis of yeast

Method of preparation as described in Section IV.

Yeast dialysate

Two hundred millilitres of a 6% yeast were autoclaved in a 250 ml. flask.

Twenty-five millilitres of sterile distilled water were introduced in a sterile dialysing bag which was immersed in the yeast extract using aseptic precautions. The flask was stored in a refrigerator at 4°C for 7 days. The yeast dialysate from the bag was pipetted into universal containers in 10 ml. portions and autoclaved.

One millilitre of yeast dialysate was added to 1 ml. of dialysed yeast in the medium.

Solution of yeast nucleic acid derivatives

A stock solution 50 x concentrated was prepared as follows:

15 mg. Guanylic acid
10 mg. Adenylic acid
12.5 mg. Cytidine
5.0 mg. Uracil

were dissolved in 10 ml. 0.1 N NaOH and autoclaved.

Co-enzyme solution

A stock solution 100 x concentrated was prepared.

3.6 mg. Co-enzyme A
5.3 mg. Triphosphopyridine nucleotide (TPN)
12.0 mg. Diphosphopyridine nucleotide (DPN)

These were all procured from Nutritional Biochemicals. The amount stated was dissolved in 50 ml. distilled water, sterilised by filtration through sintered glass, dispensed in 5 ml. aliquots and stored at -15°C.

Inorganic salts supplement

The stock solution was prepared 100 x concentrated.

250 mg. Ferrous ammonium sulphate
50 mg. Manganese chloride

were dissolved in 100 ml. distilled water and autoclaved. The colourless solution became red probably due to the formation of ferric chloride on autoclaving.

B vitamin solutions

The preparation of these solutions is described in Methods, Section I.

Results

The basal medium used in this section consisted of albumen and glucose balanced salt solution.

Firstly, a number of experiments were designed to study whether or not various protein fractions of horse serum were active in cultures. The fractions tested included equine serum albumin, alpha-, beta-, and gamma-globulins. Development in cultures containing basal + yeast + horse serum was used as controls. The amino acids supplement was not included in the controls as it was wished to discover whether part of the value of serum was the amino acids it contains (Westfall et al., 1954). The results are shown in Table 39.

When amino acids were added to the basal + yeast medium, about 60% of the flukes developed follicular testes but only 6% had active sperms. The addition of serum albumin to this medium did not increase the level of development (Table 39, ii). On the other hand, when alpha-, beta- or gamma-globulin fractions were added with amino acids to the basal + yeast medium, there was an increase in the number of flukes developing sperm and some flukes formed vitellaria and a yolk reservoir (Table 39, iii, iv & v). However, results were always much poorer than in control cultures containing horse serum.

Table 39

Shows the effect of replacing horse serum in the medium by amino acids and serum protein fractions.

	Culture no.	Additive to basal + yeast	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
			Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
(i)	105M1	amino acids	15	9	-	-	-	-	6	6.2
	105M2		10	15	1	-	-	-	6	6.2
	105M3		8	17	-	-	-	-	6	6.1
	106M1		8	17	-	-	-	-	6	6.2
	106M2		10	15	2	-	-	-	6	6.1
	106M3		1	24	1	-	-	-	6	6.2
	110M1		8	17	2	-	-	-	6	6.2
	110M2		10	15	2	-	-	-	6	6.4
	115M1		10	15	3	-	-	-	6	6.1
	115M2		9	16	5	-	-	-	6	6.1
		PERCENTAGE	36	64	6	0	0	0		
(ii)	105A1	amino acids + serum albumins	9	16	-	-	-	-	6	6.1
	105A2		11	14	-	-	-	-	6	6.1
	105A3		13	12	-	-	-	-	6	6.1
	106A1		7	18	-	-	-	-	6	6.1
	106A2		10	15	-	-	-	-	6	6.3
	106A3		4	21	1	-	-	-	6	6.2
		PERCENTAGE	36	64	1	0	0	0		
(iii)	105G1	amino acids + alpha-globulins	5	20	4	1	-	-	6	6.2
	105G2		6	19	8	5	1	-	6	6.2
	105G3		4	21	6	4	3	-	6	6.2
	106G1		1	24	13	4	1	-	6	6.1
	106G2		4	20	10	4	2	-	6	6.1
	106G3		-	25	12	6	1	-	6	6.2

Table 39 (Contd)

	Culture no.	Additive to basal + yeast	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
			Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
	110B1	amino acids + beta-globulins	2	23	12	7	4	-	6	6.3
	110B2		4	20	8	4	2	-	6	6.3
(iv)	110B3		-	25	10	7	3	-	6	6.2
	115B1		7	18	9	9	3	-	6	6.5
	115B2		-	25	12	7	3	-	6	6.2
		PERCENTAGE	10	89	41	27	12	0		
	115G1	amino acids + gamma-globulins	3	22	9	4	2	-	6	6.3
	115G2		5	20	8	2	1	-	6	6.2
(v)	$115GII_1$		7	18	5	2	2	-	6	6.8
	$115GII_2$		8	17	5	1	1	-	6	6.2
	$115GII_3$		3	22	10	7	4	-	6	6.3
		PERCENTAGE	19	81	30	13	8	0		
	105C2	horse serum	5	20	15	10	7	-	6	6.1
	105C3		-	25	15	13	11	-	7	5.8
	106C1		-	25	15	13	11	-	6	6.2
	106C2		-	25	18	16	10	-	6	6.2
(vi)	106C3		-	25	16	13	10	-	6	6.3
	110C1		3	22	15	11	9	-	6	6.4
	110C2		-	25	17	12	10	-	6	6.5
	115C1		-	25	18	14	12	-	6	6.2
	115C2		2	23	18	15	12	-	6	6.3
		PERCENTAGE	4	96	65	52	41	0		

Table 40 shows the effects of adding whole serum and dialysed serum to a medium containing basal + amino acids + B vitamins (0.025 ml. B vitamin solution I per ml., see Methods, Section I).

It was found that when serum was dialysed there was a considerable loss in its growth promoting qualities suggesting that some of the small molecular components of serum are of importance when B vitamins and amino acids are substituted for yeast extract.

Table 40

Shows the effect of adding whole serum and dialysed serum to basal (BSSG + albumen) + B vitamins + amino acids medium.

Culture no.	Additive to BSSG + alb + A.A. + B vit.	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				
8A	whole serum	10	6	2	10	8	4	3	-	12	54	7	6.8
8AV		10	4	-	8	4	4	3	-	8	24	7	7.0
8A5		10	8	2	10	8	8	6	-	10	45	7	7.0
	PERCENTAGE	100	60	13	93	67	53	40	0				
8D5	dialysed serum	6	-	-	1	-	-	-	-	7	23	7	7.2
8D6		9	-	-	-	-	-	-	-	7	21	7	7.2
8D7		10	-	-	3	-	-	-	-	8	25	6	7.4
	PERCENTAGE	83	0	0	13	0	0	0	0				

Table 41 compares the effects of the addition of yeast extract and whole serum to a medium consisting of BSSG + albumen + dialysed serum + amino acids + B vitamins (0.025 ml. B vitamin solution I per ml., see Methods, Section I).

The results confirm those of experiment 8 (Table 40) that when B vitamins and amino acids are used to replace yeast, whole serum is necessary. Dialysed serum in the absence of yeast (cultures 13C1-C3) support only the development of early testes whereas with yeast extract (cultures 13Y1-Y3) 67% of the flukes formed active sperm and 100% developed vitellaria. The addition of whole serum also improved development but the results were poorer than expected in this medium, cf. experiment 7, Table 4.

Table 41

Shows the effect of adding yeast extract and whole serum to a medium consisting of BSSG + albumen + amino acids + B vitamins + dialysed horse serum.

Culture no.	Additive to BSSG + alb+ A.A. + B vit. + dialysed serum	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubation	Final pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel-:laria	Yolk reser-:voir	Eggs				
13C1		10	-	-	-	-	-	-	-	9	32	5	7.1
13C2	none	10	-	-	-	-	-	-	-	5	16	5	7.1
13C3		10	-	-	-	-	-	-	-	8	30	6	6.9
	PERCENTAGE	100	0	0	0	0	0	0	0				
13Y1		10	10	9	10	9	10	5	-	8	40	5	7.0
13Y2	yeast extract	10	9	3	10	8	10	9	-	10	43	6	6.9
13Y3		10	8	8	10	7	10	9	-	9	42	6	7.2
	PERCENTAGE	100	90	67	100	80	100	77	0				
13S1		10	1	-	4	3	3	3	-	9	45	6	7.1
13S2	whole serum	10	-	-	6	-	3	1	-	8	30	6	7.1
13S3		10	5	2	9	4	5	1	-	10	42	5	7.0
	PERCENTAGE	100	20	7	63	23	37	17	0				

The poor development attained in BSSG + albumen + dialysed serum + amino acids + B vitamins medium (experiments 8 and 13, Tables 40 & 41) may have been due to the lower concentration of B vitamins in the medium (0.025 ml. B vitamin solution I per ml.), as it was discovered later (Section I) that a concentration of 0.05 ml. of B vitamin solution I or a high concentration of pyridoxine (50 µg/ml.) gave better development. The effects of supplementing BSSG + albumen + dialysed serum + amino acids with a B vitamin solution containing a high concentration of pyridoxine was therefore compared with that of adding yeast extract to the same medium (Table 42); also the effects of doubling the concentration of the amino acids in the B vitamin supplemented medium was studied (cultures 50E1-E3). (B vitamin solutions I and V (see Methods, Section I) were used as the vitamin supplement.)

Although sperm production in the B vitamin supplemented medium was about as good as the yeast cultures, vitellaria production was much poorer (compare cultures 49V1-V3, 50C1 and 50C3, and 49Z2-Z3, Table 42). This again confirming that when whole serum is replaced by dialysed serum there are some essential nutrients in yeast extract which are not supplied by the B vitamin

and amino acid supplement.

Development in cultures containing double the concentration of amino acids (cultures 50E1-E3) was poorer than in corresponding cultures containing normal strength of amino acids.

Table 42

Shows the effect of adding yeast extract (controls), B vitamin solution with a high pyridoxine content, amino acids + B vitamin solution with a high pyridoxine content to basal + amino acids + dialysed serum medium.

Culture no.	Additive to basal + A.A. + dialysed serum	Proportion out of 10 flukes developing genitalia								No. of flukes examined (selected)	No. in culture	Days incubation	Fin :al pH
		Testes	Mature sperm	Active sperm	Uterus	Ovary	Vitel- :laria	Yolk reser- :voir	Eggs				
49Z2	yeast extract	10	10	7	10	7	8	6	-	12	80	6	6.4
49Z3		10	10	10	10	10	10	10	-	12	60	6	6.4
	PERCENTAGE	100	100	85	100	85	90	80	0				
49V1	B vit. solution with high pyridoxine content	10	10	8	10	1	4	-	-	10	50	6	6.1
49V2		10	10	7	10	3	5	-	-	10	55	6	6.1
49V3		10	10	4	10	-	4	1	-	8	40	6	6.4
50C1		10	8	7	10	2	6	1	-	10	50	7	6.2
50C2		10	8	5	10	3	7	4	-	10	40	7	6.1
	PERCENTAGE	100	92	62	100	16	52	12	0				
50E1	A.A. + B vit. solution with high pyridoxine content	10	1	-	3	-	-	-	-	8	40	7	6.2
50E2		10	5	-	5	-	-	-	-	8	30	6	6.3
50E3		10	5	3	8	-	1	-	-	8	35	7	6.1
	PERCENTAGE	100	37	10	53	0	3	0	0				

From the results of the previous experiments (Tables 40, 41 & 42), it was deduced that the dialysable active components of horse serum were replaceable by substances in yeast extract. That is, in the presence of yeast extract, only the non-dialysable component of serum is important in the medium. In order to gain some idea as to the nature of the active small molecular component(s) of serum, it was decided to add various fractions of yeast extract, about which a fair amount was known (Table 38). It was hoped in this way to discover what substance in yeast when added to dialysed serum, would support as good development of the flukes as whole serum does. It should be added that Table 38 provides useful though not necessarily complete information as to what substances are present in yeast extract.

Preliminary experiments were first carried out to study the level of development when BSSG + albumen + dialysed serum was supplemented by (a) dialysed yeast and (b) dialysed yeast + yeast dialysate, using whole yeast extract as a control.

Development in BSSG + albumen + dialysed yeast + yeast dialysate + dialysed serum was not as good as that obtained in BSSG + albumen + whole yeast extract + dialysed serum medium. Compare cultures 75D1-D2 with

cultures 57C1-C3, 66C1-C3. There was, however, some evidence to show that yeast dialysate had some beneficial effects, since development in the BSSG + albumen + dialysed serum + dialysed yeast was improved by the addition of yeast dialysate. Compare cultures 57D1-D3, 64C1-C3 and 75D1-D2.

Table 43

Shows the effect of adding whole yeast extract, dialysed yeast and dialysed yeast + yeast dialysate to BSSG + albumen + dialysed serum.

Culture no.	Additive to BSSG + albumen + dialysed serum	No. of flukes out of 20 or 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
57D1	dialysed yeast	7	5	3	-	-	-	6	6.2
57D2		9	7	3	-	-	-	6	6.2
57D3		8	6	3	-	-	-	6	6.4
64C1		11	11	3	-	-	-	6	6.5
64C2		7	16	8	2	2	-	6	6.4
64C3		7	11	7	5	5	-	6	6.3
	Total (130)	149	56	27	7	7	0		
	PERCENTAGE	38	43	21	5	5	0		
75D1	dialysed yeast + yeast dialysate	11	14	10	8	4	-	6	6.2
75D2		5	17	8	5	4	-	6	6.2
	Total (50)	16	31	18	13	8	0		
	PERCENTAGE	32	62	36	26	16	0		
57C1	whole yeast extract	4	20	8	13	12	-	6	6.0
57C2		2	18	11	11	10	1	6	6.2
57C3		1	17	8	10	8	-	7	6.2
66C1		-	25	12	13	11	-	6	6.2
66C2		5	20	13	12	10	-	6	6.3
66C3		1	24	7	13	12	-	6	6.3
	Total (150)	13	124	59	72	63	1		
	PERCENTAGE	9	83	39	48	42	1		

Table 44 shows the level of development in BSSG + albumen + dialysed serum + dialysed yeast, and when this medium is supplemented with (a) amino acids (b) B vitamins solution with a low concentration of pyridoxine (2.0 µg/ml. medium) (c) B vitamins solution with a high concentration of pyridoxine (52.0 µg/ml. medium) (d) amino acids + B vitamin solution. Development was compared with that obtained in cultures containing dialysed serum + whole yeast + albumen + BSSG medium.

There was no evidence to show that amino acids and/or the B vitamins supplied any additional nutrient for the flukes to the medium containing dialysed serum + dialysed yeast + albumen + BSSG. The active component of yeast extract which could replace the substance(s) lost when serum was dialysed was not, therefore, a non-dialysable substance nor one of the B vitamins or amino acids tested.

Table 44

Shows the effect of adding various fractions of yeast extract to BSSG + albumen + dialysed serum.

Culture no.	Additive to BSSG + albumen + dialysed serum	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
Table 43	dialysed yeast (6 cultures)								
	PERCENTAGE	38	43	21	5	5	0		
66E1	dialysed yeast + amino acids	6	19	5	1	-	-	6	6.2
66E2		6	19	6	-	-	-	6	6.3
66E3		6	19	9	5	1	-	6	6.3
	PERCENTAGE	24	76	27	8	1	0		
64V1	dialysed yeast + B vitamins*	8	15	11	3	1	-	6	6.2
64V2		6	18	12	2	1	-	6	6.3
	PERCENTAGE	28	66	46	10	4	0		
64P1	dialysed yeast + B vitamins‡	8	14	8	1	-	-	6	6.2
64P2		4	17	7	4	4	-	6	6.5
64P3		9	12	5	-	-	-	6	6.4
	PERCENTAGE	29	57	27	7	5	0		
64S1	dialysed yeast + B vitamins‡ amnio acids	2	15	11	2	-	-	6	6.5
64S2		8	14	8	-	-	-	6	6.3
64S3		9	11	5	-	-	-	6	6.4
	PERCENTAGE	25	53	32	3	0	0		
Table 43	whole yeast extract (6 cultures)								
	PERCENTAGE	9	83	39	48	42	1		

* = low pyridoxine content to give a concentration of 2.0µg/ml. medium

‡ = high pyridoxine content to give a concentration of 52.0 ug/ml. medium

Although the addition of amino acids and B vitamins to BSSG + albumen + dialysed yeast + dialysed serum did not stimulate further development in the medium (Table 44), it was decided in later experiments to add the amino acids and B vitamins supplements to this medium as amino acids and some B vitamins have been shown to be essential nutrients of the flukes (Section I). Thus the medium containing BSSG + albumen + dialysed serum + B vitamins + amino acids formed a background medium in which the activities of other small molecular substances were measured.

Table 45 shows the degree of development when a medium consisting of BSSG + albumen + dialysed serum + dialysed yeast + B vitamins + amino acids was supplemented by (a) para amino benzoic acid and choline chloride, two vitamins present in yeast not included in the B vitamins supplement, (b) an inorganic salt supplement containing ferric and manganese ions, two elements present in yeast and known to be indispensable nutritive factors for living organisms (Baldwin, 1953), (c) derivatives of yeast nucleic acid, (d) co-enzymes known to be present in yeast.

From the results obtained in Table 45, it appeared that none of these supplements was supplying additional

growth promoting factor to the medium.

Table 46 shows in summary the effect of supplementing BSSG + albumen + yeast extract medium with amino acids + various protein fractions of serum. Development in the medium is also compared with that obtained when whole serum or dialysed serum was added to the medium.

Table 45

Shows the effect of adding various small molecular substances present in dialysate of yeast extract to a medium consisting of BSSG + albumen + dialysed serum + dialysed yeast extract + B vitamins + amino acids.

Culture no.	Additive to BSSG + alb + dialysed serum + dialysed YE + B vit. + A.A.	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
75C1	none	13	5	-	-	-	-	6	6.3
75C2		12	12	1	-	-	-	6	6.3
78C2		5	20	4	-	-	-	6	6.1
78C3		9	16	1	-	-	-	6	6.1
85C1		7	18	6	1	1	-	6	6.3
85C2		10	15	3	2	-	-	6	6.3
114A1		7	18	6	1	1	-	6	6.2
114A2		6	19	2	-	-	-	6	6.3
	PERCENTAGE	35	62	12	2	1	0		
75V1	choline + para-amino-benzoic acid	12	13	2	-	-	-	7	6.3
75V2		15	9	-	-	-	-	7	6.3
75V3		13	9	1	-	-	-	6	6.3
	PERCENTAGE	53	41	4	0	0	0		
78F1	Fe^{+++} + Mn^{++} ions	6	19	2	1	-	-	6	6.2
78F2		11	14	3	1	-	-	6	6.2
78F3		6	19	2	-	-	-	6	6.3
	PERCENTAGE	31	69	9	3	0	0		
85N1	derivatives of yeast nucleic acid	17	8	1	-	-	-	6	6.5
85N2		10	15	2	-	-	-	6	6.4
85N3		14	5	-	-	-	-	6	6.5
	PERCENTAGE	55	37	4	0	0	0		
114C1	DPN + TPN + Coenzyme A	6	19	3	1	-	-	6	6.2
114C2		2	23	3	1	-	-	6	6.2
114C3		3	22	7	3	-	-	6	6.3
	PERCENTAGE	15	85	17	7	0	0		

Table 46

The effect on development of *D. phoxini* when various protein fractions of serum with amino acids are added to BSSG + albumen + yeast extract medium compared with when whole or dialysed serum is added to the medium.

Experiment no.	Total no. of cultures	Additive to BSSG + albumen + yeast extract	Percentage of flukes developing genitalia			
			Follicular testes	Active sperm	Vitellaria	Yolk reservoir
105, 106, 110, 115 (Table 39)	9	whole serum	96	65	52	41
57, 66 (Table 43)	6	dialysed serum	83	39	48	42
105, 106 (Table 39)	6	amino acids + alpha-globulins	86	35	16	5
110, 115 (Table 39)	5	amino acids + beta-globulins	89	41	27	12
115 (Table 39)	5	amino acids + gamma-globulins	81	30	13	8
105, 106 (Table 39)	6	amino acids + serum albumins	64	1	-	-
105, 106, 110, 115 (Table 39)	10	amino acids	64	6	-	-

Discussion

In the presence of yeast extract in the medium, only the non-dialysable fraction of serum was necessary for good development (Wilson, 1960). It has been shown in Section I (this thesis) that yeast extract could be replaced partially by B vitamins and amino acids in a medium containing whole serum. However, when whole serum was replaced by dialysed serum in a B vitamin supplemented medium, there was a considerable loss of activity of the medium showing that the small molecular fraction of serum was important under these conditions (Table 40). Since the active small molecular components of serum, or substances which have the same effect, are present in yeast extract (Table 41), it may be assumed that they are water soluble and thermostable, as the aqueous yeast extract used in cultures was sterilised by autoclaving. Water soluble, heat stable constituents of yeast include B vitamins, amino acids, organic and inorganic salts, co-enzymes, carbohydrates and nucleotides.

Since it has been shown that a high concentration of pyridoxine and amino acids favoured growth (Section I), the levels of these compounds were raised in a medium containing dialysed serum to correct for their loss during dialysis of the serum. Whilst the increase of pyridoxine seemed to have no effect in the medium, it was found that

a double concentration of amino acids tended to produce some toxic effects (Table 42). The toxicity might have been produced by a few amino acids in the supplement not supplied at their right concentrations. This would raise the problem of determining a suitable range for each amino acid and also a determination of a suitable balance in their relative concentration. Before it could be concluded with certainty that it is not the loss of amino acids which retard development in dialysed yeast + dialysed serum medium, it is suggested that a mixture of the free amino acids of the type and proportion in yeast extract should be used as an amino acid supplement instead of those of tissue culture medium 703.

Since amino acids and pyridoxine were not the only dialysable, heat stable, water soluble factors in horse serum, other compounds were also tested for activity. Dialysed yeast and dialysed serum incorporated in the basal medium (BSSG + albumen) formed a background in which the contributions made by some small molecular components were assayed.

The addition of yeast dialysate to basal + dialysed yeast + dialysed serum medium increased the level of development in the medium, although development was not as good as in control cultures in which complete yeast

extract was used (Table 43). As was expected the addition of amino acids and/or B vitamins to basal + dialysed yeast + dialysed serum medium did not alter the growth properties of the medium (Table 44).

Amino acids and B vitamins were added to a medium consisting of dialysed yeast + dialysed serum + basal, in order to replace the substances lost from yeast extract on dialysis. This medium was used as a background medium in which choline and para amino benzoic acid, derivatives of yeast nucleic acid, co-enzymes, ferric and manganese ions were tested for activity (Table 45). None of these seemed to contribute nutritional properties to the medium.

From the forgoing experiments, it was concluded that serum was contributing both a dialysable and a non-dialysable component to the medium. The dialysable component is also present in yeast extract, and is water soluble and heat stable. Since in the presence of yeast extract only the non-dialysable component of serum was important, attempts were made to elucidate the active protein fractions of serum by substituting different equine proteins for horse serum in the medium. Wilson (1960) isolated various fractions of horse serum proteins by ammonium sulphate precipitation and showed that the gamma-

globulins possessed some growth promoting properties. Results obtained confirmed Wilson's findings that the gamma-globulins stimulated development, and also showed that alpha- and beta-globulins were beneficial to the flukes. On the other hand, the serum albumin fraction did not seem to possess any activity in cultures (Table 39). Hence it could be assumed that the globulin fractions of serum were responsible for part of the activity of dialysed serum in cultures.

There are reported instances in tissue culture as well as in the in vitro cultivation of nematodes that globulins are utilised more than the other protein fractions of serum. Bachtold & Gebhardt (1957) studied the quantitative utilisation of serum proteins by trypsinised cells in monkey kidney cultures grown in a defined medium supplemented with 10% calf serum. They found that all the electrophoretically separable serum components were utilised by the cells during the active growth phase and that globulins were utilised more than albumins. Similarly Kent & Gey (1957), using continuously cultured rat-tumour cells in a medium composed of 50% human placental cord serum and 50% Gey's saline, found a significant decrease in the level of alpha- and beta-globulins during a specific period of growth.

Dougherty & Keith (1953) also found that the active

factor in human plasma used as a supplement in a medium for the *in vitro* cultivation of the free living nematode, *Rhabditis briggsae*, was confined to the globulin fractions.

In none of the experiments in which globulin fractions were substituted for horse serum was development as good as those of control cultures containing horse serum (Table 39); this might indicate that proteins are not the only nutrient supplied by the non-dialysable fraction of serum in the medium. This fraction also contains lipids and sterols. It would be interesting to know the results obtained by supplementing media containing globulins with lipids or sterols.

Summary

1. Serum is supplying both dialysable and non-dialysable active components to culture media.

2. Substances which have the same effect as those in the dialysable fraction of serum are present in adequate amounts in yeast extract. As they occur in yeast extract the substances must be water soluble and heat stable.

3. The activity of the dialysable component of serum is not replaced by dialysed yeast, or by certain B vitamins, co-enzymes, nucleic acid derivatives or inorganic salts. The ambiguity of results using amino acid supplements is discussed.

4. Part of the activity of the non-dialysable component of serum is due to its globulin fractions.

5. The albumin fraction of horse serum is inactive in cultures.

6. The significance of globulins in tissue culture is briefly discussed and suggestions for future work given.

SECTION VII: ALBUMEN AS A NUTRIENT IN CULTURE MEDIA

Introduction

Of all the complex biological substances used as constituents of media for culturing flukes, albumen has been so far the most difficult to replace. It shares with serum, the property that it cannot be sterilised by autoclaving; but unlike serum native* albumen because of its viscous state cannot be sterilised by filtration either. According to Wilson (1960) the activity of albumen is almost lost on dialysis; an observation which has been confirmed in the present work. This suggests that part of the activity of albumen is due to a small molecular fraction. (It is known (Romanoff & Romanoff, 1949, pages 622-629) that albumen contains carbohydrates and also small quantities of B vitamins.) However, experiments reported here confirm Wilson's conclusions that the dialysate is also inactive. From this it may be concluded that albumen owes its activity either to a complex which is broken during dialysis, or to components such as proteins which are

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*unaltered from its natural state.

physically altered and inactivated by dilution with water. The fact that recombining the dialysate and non-dialysable fraction does not restore activity (Wilson, 1960) does not eliminate the first possibility as complexes once broken are often difficult to re-form.

The present investigations were primarily directed at ascertaining whether the activity of albumen depended on the presence of certain specific proteins, or whether hydrolysed products were equally active. Owing to the rise in temperature, and the lowering of pH in cultures it seemed probable that the proteolytic enzymes, polypetidases and dipetidases (Schulze, 1953) would in fact bring about a considerable amount of proteolysis in cultures, and this aspect was investigated by measuring the amount of amino nitrogen present after various lengths of incubation.

The other aspect considered was to what extent was it possible to replace albumen by a chemically defined supplement.

Materials and Methods

(1) Dialysed albumen

Twenty millilitres of albumen were placed in a sterile dialysing bag and dialysed against running

tap water for 73 hours. Some precipitate was formed which collected at the bottom of the bag. The clear liquid fraction was pipetted off. This liquid fraction gave positive reactions to several tests for proteins (viz. Millon's reaction, xanthoproteic reaction and precipitation by ethanol).

One millilitre of the liquid fraction was added to each culture.

(2) Dialysate of albumen

The apparatus used for the preparation of the dialysate was similar to that used for the preparation of dialysed embryo extract (figure 6). 15 ml. of egg albumen were placed in a sterile dialysing bag which was immersed in 80 ml. sterile distilled water. The albumen was changed daily, and dialysis was terminated on the seventh day. The volume of the dialysate was reduced to about 10 ml. by distillation of the water under reduced pressure at 45-50°C, and sterilised by autoclaving at 110°C for 10 minutes.

One millilitre of the dialysate was used in each culture.

(3) Homogenised seitz filtered albumen

As a means of sterilising albumen which had not been

removed from eggs aseptically, the albumen was homogenised in BSSG and seitz filtered.

Ninety millilitres of albumen obtained from three eggs were homogenised in 180 ml. BSSG in a Nelco blender at low speeds for about 5 minutes. The homogenised albumen was left standing at room temperatures to allow the foam which formed on agitation to subside. The solution was next seitz filtered. Only 60-70 ml. of sterile homogenised albumen was obtained in one hour, after which filtration was discontinued, as the pad was blocked.

Six millilitres of homogenised albumen in BSSG was used in each culture.

(4) Enzymatic hydrolysis of egg albumen

Cohn & White (1953) reported that raw albumen was almost unaffected by pepsin. After 90 minutes' incubation, they observed only 0.1% increase of amino nitrogen in a solution of albumen. Tryptic hydrolysis of albumen was also reported to be slow, but a preliminary pectic digestion of albumen greatly facilitated the hydrolysis of albumen by trypsin.

A preliminary investigation was first carried

out to discover a suitable method for carrying out enzymatic hydrolysis of raw albumen.

Thirty millilitres of fresh hen-albumen was added to 30 ml. 0.2 N HCl and shaken. The pH of the mixture was found to be 1.7-1.9. The amino nitrogen content was determined by titrating two 10 ml. portions of the solution against 0.2 N NaOH using Sorensen's method described in Section IV. 0.5 gm pepsin (1:2500) was added to the rest of the solution and the mixture incubated at 39°C. 10 ml. portions were withdrawn at intervals, and the amino nitrogen determined.

The results obtained were:

Incubation time (minutes)	Hydrolysis (ml. 0.2 N NaOH)
0	0.9
30	0.95
45	0.95
60	0.95

As very little hydrolysis occurred, the pH of the rest of the solution was adjusted to pH 8 by addition of a few millilitres of N NaOH, 0.5 gm of trypsin added and the mixture incubated at 39°C. Hydrolysis was also followed by amino nitrogen

determinations.

Incubation time (minutes)	Hydrolysis (ml. 0.2 N NaOH)
20	1.35
60	1.55
120	1.75
150	1.75

As a result of this preliminary investigation, the following standard method for hydrolysing egg albumen with enzymes was established. It should be emphasised that hydrolysis was not complete.

(a) Ninety millilitres of albumen (obtained from three eggs) were placed in a 500 ml. flask.

(b) Ninety millilitres of 0.2 N HCl were added to the albumen and the flask agitated for a few minutes. The pH of the solution was found to be 1.8.

(c) 0.8 gm pepsin (1:2500) was added and incubated for 90 minutes at 39°C.

(d) The pH was adjusted to 8.0.

(e) 0.8 gm trypsin was added, and the mixture incubated for 3 hours.

(f) Ninety millilitres of BSSG of double strength

were added to bring the final volume to 270 ml.

(g) Solution was seitz filtered. (Only 60 ml. sterile albumen solution was collected in one hour.)

(h) Six millilitres of the enzymatic hydrolysed filtered albumen were used in each culture.

(5) Peptic cleavage products of egg albumen

The method used was similar to that described by Carrel & Baker (1926).

(a) Ninety millilitres of albumen were dissolved in 90 ml. 0.2 N HCl. The amino nitrogen content determined.

(b) 0.8 gm pepsin added, and the mixture incubated for 2 hours.

(c) The solution adjusted to pH 7.0-7.2 and its volume reduced to about 40 ml. and filtered. (The boiling was designed to destroy the pepsin and the filtration to remove heat precipitated proteins.)

(d) The cleavage products were sterilised by autoclaving at 110°C for 10 minutes.

(e) 1.5 ml. was used in each culture.

(6) The determination of amino nitrogen in egg albumen after different periods of incubation at 40°C

As albumen is known to contain polypeptidases and dipetidases (Schulze, 1953), it was decided to discover whether there was an increase of amino nitrogen in a medium containing BSS + albumen on incubation.

About 60 ml. of albumen obtained from two eggs were dissolved in about 120 ml. BSS in a 250 ml. flask. The flask was shaken for a few minutes, during which time the pH was adjusted to 6.8-7.0. 5,000 units of penicillin and 0.25 gm streptomysin were added and the solution dispensed in 22-25 ml. aliquots in universal containers, and incubated at 40°C with intermittent shaking. Aseptic precautions were observed throughout. The amino nitrogen content was determined at various intervals during incubation.

The results obtained were:

Table 47

Time (hours)	Hydrolysis ml. 0.5 N NaOH
0	2.5
15	2.75
20	2.8
40	2.9
70	2.92
90	2.9

Results

As albumen, a largely proteinaceous substance was known to stimulate early genital development in D. phoxini (Bell, 1958), it was decided to investigate whether the addition of amino acids to BSSG would have the same effect. Table 48 shows the level of development reached by the flukes when amino acids and/or albumen was added to BSSG.

In the BSSG medium alone (cultures 111C1-C3) there was no development and only 20% of the metacercariae were alive after 4 days' incubation. In the BSSG + amino acids medium, the flukes were all found dead after 4 days' incubation. When albumen was added to BSSG, only 3% of the flukes developed early testes in the medium. On the addition of amino acids to the latter medium, there was an increase in the number of flukes with early testes.

Table 48

Level of development obtained in (a) BSSG, (b) BSSG + amino acids, (c) BSSG + albumen and (d) BSSG + albumen + amino acids.

Culture no.	Additive to BSSG	No. of flukes out of 25 taken at random, developing genitalia			Final pH	Days incubated
		Genital primodium	Early testes	Follicular testes		
111C1	none	ABOUT 20% OF THE METACERCARIAE WERE STILL ALIVE IN EACH CULTURE			6.5	4
111C2					6.3	4
111C3					6.1	4
111A1	amino acids	FLUKES ALL DEAD			6.3	4
111A2					6.2	4
111A3					6.2	4
77B1	albumen	6	1	-	6.3	6
77B2		7	1	-	6.2	6
77B3		5	-	-	6.3	6
	PERCENTAGE	24	3	0		
77BA1	albumen + amino acids	8	9	-	6.2	6
77BA2		9	7	-	6.2	6
77BA3		9	9	-	6.0	6
	PERCENTAGE	36	35	0		

In experiment 116 an attempt was made to discover to what extent the activity of albumen was lost or augmented by certain analytical procedures. In cultures 116P1-P3, pepsin digested albumen was used, in cultures 116D1-D3, the dialysate and in cultures 116A1-A3 the non-dialysable fraction. Cultures containing BSSG + albumen were used as controls. The results are summarised in Table 49.

Neither the peptic products not the dialysate of egg albumen stimulated growth or even increased survival above the level in BSSG. The addition of dialysed egg albumen increased survival but did not stimulate growth; this would suggest that the proteins of albumen had some beneficial effects in cultures.

Table 49

Shows the effect of adding albumen (controls), peptic cleavage products of albumen, dialysate of albumen and dialysed albumen to BSSG.

Culture no.	Additive to BSSG	% of flukes surviving after 5 days incubation	No. of flukes out of 25 taken at random, developing genitalia			Final pH
			Genital primodium	Early testes	Follicular testes	
116B1	none	8	-	-	-	6.3
116B2		8	-	-	-	6.2
116B3		8	-	-	-	6.1
116P1	peptic cleavage products of egg albumen	6	-	-	-	6.4
116P2		5	-	-	-	6.3
116P3		8	-	-	-	-
116D1	dialysate of egg albumen	5	-	-	-	6.4
116D2		5	-	-	-	6.3
116D3		3	-	-	-	6.3
116A1	dialysed egg albumen	36	-	-	-	6.4
116A2		28	-	-	-	6.3
116A3		39	-	-	-	6.3
116C1	native albumen	75	7	2	-	6.2
116C2		75	5	-	-	6.2
116C3		87	9	1	-	6.3

In order to be able to test various fractions of albumen produced as a result of applying certain analytical procedures, most of which contain heat labile substances, it was first essential to discover a method of sterilising other than by heat. As mentioned in the introduction native albumen is too viscous to filter but very dilute solutions can be filtered without losing their power to promote the growth of Entamoeba at least (Hoare, 1949, page 305-306).

As a result of various preliminary experiments it was found that 33% albumen in BSSG could be filtered and as is shown in Table 50 little or no loss in its activity to promote growth of D. phoxini was observed.

Table 50

Shows the effect of adding native albumen (controls) and seitz filtered homogenised albumen to BSSG + amino acids + yeast extract + horse serum medium.

Culture no.	Additive to BSSG + A.A. + YE + HS	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				
11K1	seitz filtered homogenised albumen	10	10	7	10	10	10	8	-	10	43	5	7.0
11K2		10	10	9	10	10	10	10	1	8	24	5	7.0
	PERCENTAGE	100	100	80	100	100	100	90	5				
11M1	native albumen	10	10	6	10	10	10	10	1	10	41	5	7.1
11M2		10	10	10	10	10	10	10	2	10	51	5	7.0
	PERCENTAGE	100	100	80	100	100	100	100	15				

Albumen shows considerable growth promoting effects when added to a medium containing BSSG + yeast extract + horse serum (Table 51, cf. cultures 117B1-B3 and 117C1-C2). In order to discover the active fraction of albumen, the effects of supplementing BSSG + yeast + horse serum medium with (a) dialysed albumen, (b) enzymatic hydrolysed albumen are compared with those of supplementing the medium with native albumen (Table 51).

There was almost a complete loss of activity when native albumen was replaced by either dialysed or enzymatic hydrolysed albumen in the medium.

Table 51

Shows the effect of adding albumen (controls), dialysed albumen and enzymatic hydrolysed albumen to BSSG + yeast extract + horse serum medium.

Culture no.	Additive to BSSG + yeast extract + horse serum	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
117B1	none	9	16	3	4	3	-	6	6.4
117B2		13	12	2	1	1	-	6	6.3
117B3		13	12	3	2	1	-	6	6.3
	PERCENTAGE	47	53	11	9	7	0		
117D1	dialysed albumen	7	18	6	1	-	-	6	-
117D2		10	15	3	-	-	-	6	6.3
117D3		12	13	3	3	3	-	6	6.2
	PERCENTAGE	38	61	16	5	4	0		
102E1	enzymatic hydrolysed albumen	6	19	5	3	3	-	6	6.6
102E2		2	23	8	7	4	-	6	6.6
102E3		8	17	3	1	1	-	6	6.5
	PERCENTAGE	21	79	21	15	10	0		
102C1	native albumen	-	25	7	7	7	-	6	6.4
102C2		1	24	10	10	10	-	6	6.4
117C1		3	22	17	11	9	-	6	6.3
117C2		3	22	17	16	12	-	6	6.4
	PERCENTAGE	7	93	51	44	38	0		

Table 52 shows development of the flukes in BSSG + yeast + horse serum medium and also when the medium was supplemented by (a) albumen, (b) amino acids.

Development in BSSG + yeast + horse serum medium was poor, only about 10% of the flukes developed active sperms and vitellaria; on supplementing the medium with albumen there was a considerable increase in the number of flukes producing sperms and vitellaria. Only about half of the activity of albumen was obtained when the medium was supplemented with amino acids.

Table 52

A comparison of the level of development obtained when (a) albumen and (b) amino acids were added to a basal medium consisting of BSSG + yeast extract + horse serum.

Culture no.	Additive to BSSG + yeast extract + horse serum	No. of flukes out of 25 taken at random, developing genitalia					
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs
Table 51	none (3 cultures)						
	PERCENTAGE	47	53	11	9	7	-
Table 51	albumen (4 cultures)						
	PERCENTAGE	7	93	51	44	38	-
98B1	amino acids	-	25	8	6	5	-
98B2		-	25	9	8	4	-
98B3		3	22	7	2	2	-
	PERCENTAGE	4	96	32	21	15	0

Table 53 compares level of development when (a) no supplement, (b) amino acids, (c) albumen, (d) albumen + amino acids were added to a medium consisting of BSSG + B vitamins + horse serum.

In a medium containing BSSG + B vitamins + horse serum, very little development occurred; only a very few flukes developed follicular testes. On the addition of amino acids to this medium, about half of the flukes in each culture showed varying degrees of development; a few developed active sperms, vitellaria and yolk reservoirs and others had follicular testes. However, it was surprising to find a large number of dead flukes in each culture. Development in the latter cultures (i.e. those with amino acids but no albumen)was not as good as that in which albumen was added to the BSSG + B vitamins + horse serum medium. Very few dead flukes were found in these cultures. When both albumen and amino acids were added to the medium, the level of development was greater than when each was added separately. The apparent toxic effects of the amino acids appeared to be suppressed in the presence of albumen as only a few dead flukes were found in these cultures.

Table 53

A comparison of the level of development when (a) no supplement, (b) amino acids, (c) albumen and (d) albumen + amino acids were added to the BSSG + B vitamins + horse serum medium.

Culture no.	Additive to BSSG + B vitamins* + horse serum	No. of flukes out of 25 taken at random, developing genitalia						Days incubated	Final pH
		Early testes	Follicular testes	Active sperm	Vitellaria	Yolk reservoir	Eggs		
Table 10	none (3 cultures)								
	PERCENTAGE	65	27	1	3	1			
82C2	amino acids	13	10	1	1	1	-	6	6.3
82C3		11	11	3	1	1	-	6	6.3
	PERCENTAGE	48	42	8	4	4	0		
63C1	albumen	13	10	2	2	2	-	6	6.6
63C2		5	20	5	4	3	-	6	6.4
	PERCENTAGE	36	60	14	16	10	0		
91V1	albumen + amino acids	6	19	6	8	8	-	6	6.3
91V3		5	20	6	12	11	-	6	6.3
	PERCENTAGE	22	78	24	40	38	0		

*Final concentration of pyridoxine was 52.0 µg/ml. medium.

Discussion

Wilson (1960) using a basal medium of yeast extract + horse serum + BSSG showed that amino acids gave part of the activity of albumen when used as a supplement to the basal medium. She also observed that a higher level of development was obtained when the basal medium was supplemented with both albumen and amino acids. The results obtained in Table 53 are in accordance with those obtained by Wilson although the yeast extract which she used in the basal medium was in this case replaced by B vitamins. In a medium consisting of BSSG + B vitamins + amino acids + horse serum, only 8% of the flukes developed active sperms and about 4% vitellaria and yolk reservoirs. All the constituents except those of serum in the medium are chemically defined. The poor results obtained in this medium, could be attributed to many factors, but chiefly to an apparent toxic effect produced by amino acids in the absence of albumen in the medium. The toxic effects produced by the amino acids are more evident in experiment 111 (Table 48) in which all the flukes in cultures containing BSSG + amino acids were dead after 4 days' incubation, whilst at least 20% of the flukes in control cultures containing BSSG survived during the same period. This toxic effect is probably due to a metabolic imbalance produced by some unfavourable

levels of amino acids in the supplement. Thus it is not yet possible to tell whether the flukes require protein in their diet, or whether amino acids might supply the need, if provided in correct balance.

Development was improved when amino acids were added to a medium consisting of BSSG + albumen (Table 48). Since the albumen in each culture when hydrolysed yields over twelve times as much amino acids - 200 mg. (Romanoff & Romanoff, 1949, p.319) as that added in the amino acid supplement (16 mg. of medium 703 amino acids) it was surprising to find that the amino acids stimulated development as much as they did. However, this assumes that the flukes can hydrolyse albumen, an assumption reasonable in the case of a free living animal, but less justifiable when considering an intestinal parasite. A probable explanation is that the flukes do not secrete the necessary enzymes to break down the proteins extracellularly in order to absorb them as amino acids. Since albumen contains a small quantity of amino acids and smaller protein breakdown products, it would not be unreasonable to assume that the flukes use only amino acids and peptides in albumen.

Albumen also contains a number of peptidases; according to van Manen & Rimington (1953), two erepsins

are present in albumen, one with a pH optimum of 5.5, the other with a pH optimum of 7.0-8.0. These authors found no true proteinase in the thick or thin fractions of albumen; Schultze (1943) reported that albumen contained both polypeptidases and dipeptidases. It would seem probable that slow digestion of proteose in albumen to utilisable amino acids occurs during incubation. When amino acids are, however, added as supplement to BSSG + albumen medium, the quantity of available amino nitrogen is increased and this resulted in a corresponding increase in the level of development. Investigation of the rate of production of amino nitrogen in a 50% albumen in BSSG on incubation (Table 47) revealed that there is an increase of about 17% of amino nitrogen after 40 hours' incubation. It must be emphasised that this value obtained is only approximate. It is probable that the use of other techniques, e.g. gravimetric, colorimetric and microbiological procedures might give more accurate results.

In Table 49, it was demonstrated that neither the addition of peptic cleavage products of egg albumen nor the dialysate of egg albumen containing carbohydrates, B vitamins and small molecular nitrogenous products of albumen to BSSG stimulated growth or increased survival of the flukes. However, it was interesting to find that

survival of the flukes in BSSG + dialysed albumen showed an improvement over that in BSSG alone. Although about a third of the flukes survived after 5 days' incubation, the flukes did not proceed beyond the metacercarial stage. Survival was still poor when compared with that in BSSG + albumen medium in which there was over 80% survival. Wilson (1960) suggested that one of the probable roles of albumen in the medium was detoxification. The increase survival of the flukes in BSSG + dialysed albumen over that in BSSG seem to justify her views; it is probable that waste products of the worms inhibited survival in BSSG, the effects of the dialysed albumen was to inactivate the toxic waste products. It is an established fact that albumins produce detoxifying effects in tissue cultures; one may quote the work of Jacquex & Barry (1951) who demonstrated the action of serum albumin in detoxifying oleic acid liberated in cultures by rat fibroblasts.

Willmer & Kendal (1932) showed that the growth of chicken heart fibroblast in tyrode was not increased on the addition of proteoses, but in the presence of plasma, the addition of proteoses causes increased growth of the tissues probably owing to slow digestion of proteins to utilisable amino acids. Results shown in Table 49 indicate that the flukes were also unable to utilise dialysed albumen in BSSG. It was decided, therefore,

to determine whether in a basal medium containing BSSG + yeast extract + serum the flukes could use enzymatic hydrolysed egg albumen and dialysed albumen. Results obtained in Table 51 indicated that the flukes were unable to use either dialysed albumen or enzymatic hydrolysed albumen; this eliminates the possibility that there might be breakdown of albumen by enzymes in the serum, to produce amino acids which are beneficial to the flukes.

Considering the various analytical procedures which have been applied to egg albumen, viz. solidification by heat (Hopkins, unpublished), precipitation of some of the proteins, and dialysis (Wilson, 1960), enzymatic hydrolysis and homogenising in BSSG followed by seitz filtration; it was only in the case of the last mentioned process that the albumen retained its activity after the treatment (Table 50). The activity of the albumen was almost lost when the other procedures were applied. This might possibly indicate that albumen possesses some labile nutritive factors. It may be, that the process of heating, precipitating, hydrolysing by enzymes and dialysing destroy some peptidases which are capable in the whole (native) albumen of breaking down small polypeptides to amino acids, which in turn supply an essential requirement of the flukes. The failure of the amino acid supplement

of medium 703 to give as good a result as albumen when added to the BSSG + yeast + horse serum medium (Table 52) can be attributed to a metabolic imbalance produced by some amino acids in the supplement and also probably to an inadequate supply of amino nitrogen in the medium. It is suggested for future work that the free amino acids of the type and proportion in egg albumen should be tested as an amino acid supplement instead of those of tissue culture medium 703. It would be relevant in this respect to quote the work of Timms & Bueding (1959) who found that the free amino acids, of the type and proportion in globin, were more effective in prolonging the survival of Schistosoma mansoni in vitro than globin itself, or other mixtures of amino acids. These workers were able to demonstrate the presence of a proteolytic enzyme with a high specificity for haemoglobin in the worms.

Summary

1. The amino acids solution of tissue culture medium 703 partially replaces the effects of albumen when added to a basal medium containing horse serum + BSSG + yeast extract or B vitamins. Some toxic effects produced by the amino acids are completely suppressed in the presence of albumen in which the nutritive effects of the amino acids are still expressed.

2. The toxic effects of the amino acids are evident when flukes were cultured in BSSG + albumen.

3. The survival of the flukes in BSSG is increased on the addition of dialysed albumen. The probable role of the dialysed albumen in the medium is discussed.

4. Dialysis and enzymatic hydrolysis of albumen greatly altered its activity in culture media. It is suggested that these procedures destroy the associated enzymes contained in albumen.

5. There is an increase of amino nitrogen in a saline medium supplemented by albumen on incubation.

6. Suggestions for future work and the probable role of albumen in the medium are discussed.

SECTION VIII: THE IN VITRO CULTIVATION OF FLUKES FROM INTESTINE OF DUCKLINGS

Introduction

During the study of the replacement of biological complex materials by suitably defined components in culture media, it was noticed that a great variation in the level of development by individual flukes occurred in almost every culture; for example, in a culture containing a good medium such as BSSG + yeast extract + amino acid + albumen + horse serum, it was common to find a few flukes producing active sperms, vitellaria, yolk reservoirs and abnormal eggs, whilst others would scarcely have passed the metacercarial stage and the rest showing intermediate stages of development. As some of the variability in the developmental stages reached by the flukes may be attributable to an intrinsic capacity of the metacercaria to develop, it was decided to try to overcome such variation in cultures by cultivating only those flukes which have been shown to be infective to a suitable laboratory host. Metacercariae were fed to ducklings, and by carrying out autopsies at suitable intervals, flukes were removed at different stages of their development and transferred into culture media.

Since the late maturation stages in the flukes,

resulting in the production of genitalia and egg-shell precursors have been described for the flukes developing in ducklings (Bell & Smyth, 1958) it was also decided to study whether or not the flukes which have already formed the precursor of egg-shell materials in the duck, would produce normal eggs if transferred to cultures, as failure of the metacercaria in culture media to produce normal egg-shell has always been associated with some nutritional deficiency (Bell, 1958; Smyth, 1959).

Materials and Methods

Metacercariae of Diplostomum phoxini were obtained from the brain of minnows; adult trematodes were obtained by feeding whole minnow heads to ducklings; by performing autopsies at suitable intervals, worms in all stages of development from metacercaria to adult could be obtained. Oviposition takes place between 60-70 hours in the duck (Bell & Smyth, 1958). The flukes were next transferred to sterile saline at 39°C, washed with several changes of sterile saline containing antibiotics before being transferred to culture media. Incubation was then continued, with intermittent shaking at 39°C. The medium was changed every 48 hours. Aseptic precautions were observed where possible.

Medium

The medium used consisted of glucose balanced salt solution (BSSG); horse serum - HS (30%); albumen - alb, (12½%); yeast extract, (5 mg. per ml.); amino acids - A.A. (see Section I); penicillin (10 units per ml.); and streptomycin (50 units per ml.).

Method of assessing development

Only in a few cultures was detailed examination of all the flukes done. In the rest only the number of flukes with eggs and the number and nature of the eggs picked up in the medium were recorded.

Diazo test

The diazo technique, described by Johri & Smyth (1956) was used to detect the nature of the egg-shell precursors in the vitellaria of the flukes.

Results

The results in this section are summarised in Table 54. The first and second vertical columns in the table record the length of development of metacercariae in the duck and the genitalia formed during the period. The third gives duration of incubation in cultures. The fourth and fifth contain the number of flukes placed in each culture and the number recovered alive after incubation respectively. The number of eggs recovered from cultures corrected when over ten to the nearest ten is shown in column 6. As most of the eggs recovered from cultures were abnormally shaped and darkly tanned, a count was made of the few which appeared normal, this is expressed as a percentage of the total number of eggs in the medium in column 7.

Cultivation of flukes after 27 hours in duck

Flukes recovered from ducklings 27 hours after feeding the metacercariae had developed good follicular testes, but no active sperm was observed in any of the six examined. Of 24 flukes incubated in the yeast medium in one culture, 20 survived 5 days; the medium was changed every second day. No further maturation had occurred in 6 of the flukes, which showed only testes. Two flukes developed well forming active sperms,

vitellaria, yolk reservoir and one egg each. The other 13 showed intermediate stages of development.

In another culture where incubation was extended to 7 days, survival was poorer, only 8 flukes out of 20 placed in the culture were alive. A similar variation in the level of development attained by individual flukes was observed.

Cultivation of flukes after 50 hours in the duck

Well developed testes producing active sperm and a few vitelline cells were found in flukes which had been in the duck for 50 hours. Flukes were transferred into cultures and examined after 48 hours' incubation. Survival was good and about 40% of the flukes in each culture had at least one egg in the uterus. No shell was observed in any of the eggs. A few eggs were recovered from the medium; these failed to hatch after 13 days' incubation at 26°C. A diazo test (Johri & Smyth, 1956) was applied to 4 of the flukes. Only one with a widely distended yolk reservoir gave a positive reaction. In the other 3, scattered patches of vitellaria appeared orange-red whilst most of the vitellaria gave orange-yellow colouration with the diazo reagent.

Cultivation of flukes after 66 hours in the duck

Sixty-six hour flukes recovered from the duck's

intestine were found to have fully developed genitalia and to have started producing eggs. Those without eggs in the uterus were incubated in two cultures. All the flukes were alive in the first 24 hours' incubation (Table 54.3). Only 70% of the flukes had eggs in the uterus. Most of the eggs picked up from the medium were improperly shaped and darkly tanned; only five appeared normal, three of which showed signs of embryonation after 5 days' incubation, but all the eggs failed to hatch after 13 days' incubation at 26°C.

In the second culture, the flukes continued to produce eggs after 96 hours' incubation. The medium in this culture was changed every 48 hours and incubation continued till the eighth day. About 70% of the flukes were still alive although egg production was considerably reduced.

Flukes recovered from the duck with eggs in the uterus were incubated in one culture. All the eggs picked up from the medium after 24 hours' incubation appeared normal (Table 54.4). Some of these eggs would be those in the uterus of the flukes at the time of transfer from the duck. After a further period of 48 hours, all the eggs in the culture were abnormal.

Table 54

Survival and egg production in cultures of flukes recovered from intestine of ducklings.

	No. of hours in duckling	Genital structure already formed	Days of incubation in yeast medium	No. of flukes placed in culture	No. of flukes recovered alive	Eggs in medium	
						Total	% apparently normal
1	27	follicular testes	5	24	21	3	0
			7	20	8	0	0
2	50	testes with sperms, ovary and a few vitelline cells	2	20	20	20	0
			2	50	45	50	0
			2	7	5	20	10
3	66	testes with sperms, ovary, vitellaria and yolk reservoir (no eggs)	1	10	10	60	10
			4	7	7	70	0
			8	7	5	20	0
4	66	testes with sperms, ovary, vitellaria and yolk reservoir and eggs	1	25	25	200	100
			3	25	25	60	0
			8	25	9	20	0

Discussion

In the metacercaria of Diplostomum phoxini only a minute gut, suckers, adhesive organ and excretory vesicle are present. On ingestion by a warm-blooded host further development is probably initiated by the sudden rise in temperature and characterised by an outburst of intense mitotic activity (Bell & Hopkins, 1956) resulting in the development of follicular testes. When these flukes were transferred into culture media at 39°C they continued maturation, but the level of development varied considerably; whilst little further maturation occurred in some; in a few active sperms, vitellaria, yolk reservoir and abnormal eggs developed; the rest showed intermediate stages of development. Since conditions in cultures are different from those in the intestine of the duck, it would seem that only those flukes which can adjust themselves to the new environment in culture media, were able to continue maturation. The level of development reached by each metacercaria in a given medium must therefore depend on its intrinsic capacity to develop under the given conditions.

Fifty hour flukes with well developed testes, active sperms, ovary and a few vitelline cells continued their development in culture medium. Using the diazo technique

of Johri & Smyth (1956), it was possible to show in some of the flukes that a number of the vitelline cells were abnormal. If the vitelline cells which gave positive diazo reaction were formed in the duck, then the abnormal synthesis of vitellaria and egg-shell precursors might possibly be correlated with some nutritional difficiences in the medium.

Sixty-six hour flukes recovered from the duck were found to have fully developed genitalia and to have started producing eggs. It was surprising to find that about 90% of the eggs produced by such flukes in cultures after 24 hours were abnormal, since normal synthesis of precursor of egg-shell material would have occurred in the flukes in the intestine. The failure of these flukes to continue producing viable eggs in cultures suggests that their metabolic activities are not maintained at the 'normal' adult level. Similarly it is probable that the metabolism of the flukes during development in vitro is quite different from that in vivo, since flukes which had not completed their maturation in vivo (i.e. 27-hour and 50-hour flukes) also produced abnormal eggs in cultures.

Two probable explanations are given to account for the apparent difference of metabolism in vivo and in cultures.

(i) The culture medium is deficient in some dietary essentials; and/or, (ii) the physico-chemical character of the medium is quite different from that of the host environment, and unsuitable for the normal development of the flukes. The problem now raised of course, is that of providing a medium with a suitable physico-chemical characteristic simulating that of the intestine of the definitive host and at the same time satisfying the nutritional requirements of the flukes.

Summary

1. Diplostomum phoxini in various stages of adult development were recovered from the intestine of ducklings and transferred to culture media.

2. Flukes after 27 hours and 50 hours in the duck respectively continued maturation in vitro. There is a variation in the level of development attained by individual flukes, in no case are normal eggs formed.

3. Adult flukes recovered from the duck after 66 hours' incubation, produce normal eggs for 24 hours but abnormal thereafter.

4. Probable reasons for the failure of the flukes to produce normal eggs in cultures are given.

CONCLUSIONS

In vitro cultivation provides a suitable experimental method for investigating the ways in which the gut environment controls the maturation and development of *D. phoxini*. The goal at which to aim is a chemically defined medium kept under known physical conditions. Little information can be obtained on the nutrition of the worms *in vitro* so long as it is necessary to use complex biological materials in the medium, such as serum, albumen, yeast extract, liver extract and embryo extract, in order to obtain any significant level of development. Not only are these substances complex, but they are also variable. Serum, for example is obtained from different animals whose metabolism may differ considerably; to mention two such differences, the globulin fraction will vary depending on the antigen history of the animal and the enzymes differ in total concentration or in balance of iso-enzymes (Wroblewski, 1961). Indeed it has long been known to workers in the field of tissue culture and more recently virologists that one batch of serum may support good growth whereas another does not.

The discovery that *D. phoxini* matures to the extent of producing active sperms and abnormal eggs in a medium

consisting of glucose balanced salt solution (BSSG) + amino acids + three undefined components, viz. albumen, horse serum and yeast extract (Wyllie et al., 1960) poses many interesting problems. For instance, are the undefined components suitable because they provide bulk or trace metabolites, or a well buffered physical environment, or because they detoxify waste products produced by the worms. The problem was investigated in two ways: (a) attempts were made to replace serum, albumen and yeast extract by simpler natural products which could be analysed more easily; (b) each undefined component (i.e. serum, albumen and yeast extract) was analysed in an attempt to identify the important constituent.

Autoclaved liver extract was found to have an equal growth promoting effect as yeast extract when added to the other constituents of the medium (i.e. BSSG + HS + alb). Thus it can be concluded that both yeast and liver extracts are contributing thermostable factors to the medium. Although unheated yeast extract gave quite as good results as the autoclaved extract (Wilson, 1960), raw liver extract gave only part of the activity of the heated extract. This suggests that either the activity of the growth promoting substances in liver increases on autoclaving or that raw liver extract

contains a toxic factor which, however, is destroyed by heat. The latter explanation seems more likely as it was later discovered that in the absence of serum in the medium, the addition of raw liver extract to the medium seemed to introduce a lethal factor.

In the presence of the other medium constituents, extracts of 11-day chick embryos appeared to be a suitable replacement for horse serum in the medium. Like serum most of the activity of the extract is confined to its non-dialysable fraction, suggesting that the active components are large molecular compounds, e.g. proteins or small molecular bound or incorporated in proteins. Although the action of heat on the growth promoting substances in serum are not known, the activity of embryo is almost completely destroyed by heating to 80°C for 10 minutes.

Although successful natural products were discovered which could replace serum and yeast in the medium, it was observed that these substances were as complex as those they were designed to replace. As the replacement of horse serum by embryo extract, or yeast by liver extract did not produce better development, and as a considerable amount was already known about the effects of different fractions of yeast and serum, it was decided to postpone

further investigation of embryo and liver extracts and to apply analytical procedures to yeast and serum.

Yeast extract was found to be contributing (a) a dialysable fraction and (b) a large molecular non-dialysable fraction. The active part of the dialysable fraction consisted mainly of amino acids; whilst part of the non-dialysable component of yeast appeared to be a small molecular substance, resistant to mild acid and alkaline hydrolysis, basically charged and usually occurring in a large non-dialysable complex. This substance was found to be replaceable in the medium by pyridoxine or some other form of vitamin B_6. Vitamin B_6 occurs as a non-dialysable protein complex in yeast (Kuhn & Wendt, 1938) is basically charged and resistant to 5N acid and alkaline at 100°C (Robinson, 1951). Supplementing BSSG + albumen + horse serum + amino acids medium with the B vitamin supplement of Waymouth's Medium M.B. 752/1 (quoted by Paul, 1951) considerably improved development though the results were poorer than those obtained with yeast. The interesting point, however, was that vitamin B_6 gave as good results as the whole vitamin supplement. While there is no doubt that B_6 permits more development to take place, it does so only at a concentration far exceeding that in yeast. The conclusion is, therefore, that there are some substances

in yeast extract other than those considered in this work which are used by the flukes, the effect of which can be partially replaced by a high concentration of pyridoxine.

In the presence of yeast extract amongst other constituents in the medium, the dialysable substances of serum are of no importance; but on replacing yeast extract by B vitamins and amino acids, it was discovered that at least some of the dialysable substances of serum were utilised by the flukes. It follows from these observations that there must be substances in the dialysate of serum which also occur in yeast extract (or have the same effect as substances in the yeast extract). Since the yeast extract components are water soluble and thermostable, the active small molecular fraction lost when serum is dialysed is also likely to be water soluble and thermostable. Although a variety of the substances known to occur in yeast extract were tested for example, mineral salts, nucleic acid derivatives, no suitably defined component was discovered as a substitute for the active dialysable substances of serum.

Of various protein fractions of horse serum tested for activity in a medium containing BSSG + albumen + yeast + amino acids, only the globulin fractions were found to

be active. The alpha-, beta- and gamma-globulin fractions gave almost equal activity. In no case were the results as good as that obtained when the medium was supplemented with whole serum; suggesting that the non-dialysable fraction of serum was contributing some components other than globulins to the medium, or the particular forms of the globulins were important.

Egg albumen was partly replaceable by amino acids in the medium. It was therefore concluded that albumen was at least a source of nitrogen in the medium. It was found that there was a slight increase in amino nitrogen in a medium containing albumen + BSSG during incubation at 40°C. This suggests that the proteins of albumen, presumably because of its associated enzymes, undergo proteolysis to yield free amino acids and peptides.

Although the addition of the amino acid supplement to cultures produces some beneficial effects, according to Wilson (1960), the amino acids in the absence of albumen in the medium tend to introduce some toxic effects as well; an observation which has been confirmed in the present work. This shows that albumen is performing a role other than that of supplying additional nutrients to the medium. Nicholas et al. (1959), in the axenic cultivation of the free living nematode, Caenorhabditis briggsae, suggested the following hypotheses for the

role, biological complex materials played as supplements to defined basal media; (1) that the supplements are needed to supply one or more nutrients not in the basal media (additional hypothesis), (2) that such media contain all the necessary nutrients, but are incorrectly balanced, in which case the supplement must correct this imbalance (balance hypothesis), (3) that the supplements detoxify some waste material produced by the worms (detoxifying hypothesis) and (4) that the supplements facilitate the assimilation of one or more nutrients in the basal medium (assimilation hypothesis). These hypotheses are now considered in the light of results obtained by supplementing different media with dialysed and whole albumen.

Additional nutrient hypothesis

The fact that albumen is contributing additional nutrients to media has been well established (Wilson, 1960).

Balance hypothesis

It was discovered that flukes survived for a longer period in BSSG than in BSSG + amino acids; but on supplementing both media with albumen, the level of development in BSSG + amino acids + albumen was higher than that obtained in BSSG + albumen demonstrating the

beneficial effects of the amino acids. The shorter survival of flukes in BSSG + amino acids medium than in BSSG might be attributed to a metabolic imbalance produced by a toxic ratio or level of certain amino acids in the medium. Albumen might be correcting this metabolic imbalance, as well as performing other functions, and hence not only was survival increased, but also growth was stimulated when the medium was supplemented with albumen.

Detoxifying hypothesis

D. phoxini can survive for a longer period in BSSG + whole albumen or dialysed albumen than in BSSG alone. The early death of the flukes in BSSG could probably be due to a toxicity produced by the accumulation of the waste products of metabolism of the worms. Whole albumen or dialysed albumen which is not growth stimulating, might be detoxifying the waste products of the flukes when used as supplements and hence survival is increased. Jacquez & Barry (1951) have demonstrated the action of serum albumin in detoxifying oleic acid liberated in cultures by rat fibroblasts.

Assimilation hypothesis

It has been shown that serum albumin can make oleic acid available to the bacterium, Myobacterium tuberculosis

(Davis & Dubois, 1947). It is possible that the addition of albumen to media also assisted in making some of the components of the medium available to the flukes; but as yet there is no evidence to support this.

The above hypotheses were examplified by results obtained with albumen used as a supplement. It is also possible that the other two biological complex substances in the medium, namely serum and yeast extract are to some extent capable of performing the functions discussed. In addition to the four probable functions mentioned by Nicholas et al. (1959), the addition of biological complex substances to defined media have been shown to produce a well buffered physical environment; for example, Bell & Hopkins (1956) demonstrated that the addition of horse serum to BSSG provided a well buffered solution in which D. phoxini survived well.

Results so far obtained in the elucidation of the nutritional requirements of D. phoxini in vitro show that the flukes appear to require almost all the main classes of nutrients of higher animals; namely carbohydrates (Bell & Hopkins, 1956), vitamins, amino acids and proteins. It is not yet possible to demonstrate a lipid requirement of the flukes. This is probably due to the failure to supply lipoid nutrients

in media in a form in which they are readily available to the flukes. An alternative explanation is that the flukes can synthesise their fats and sterols from carbohydrates and hence do not need an exogenous supply of fatty substances.

Some of the work described in this thesis is no more than a preliminary trial of experiments which could be repeated with improved techniques. For example, the characterisation of the small molecular components of serum could be done by separating the components of serum by ultrafiltration and a detailed analysis of the ultrafiltrate carried out. With the use of specific molecular sieves it would be possible to determine the approximate molecular weight of the active components. The application of analytical procedures to liver and embryo extracts may also reveal useful information on the nutrition of the flukes.

The greatest difficulty encountered when assessing media was the variation in the level of development of flukes in cultures. Even in supposedly identical control cultures containing a good medium consisting of BSSG + yeast extract + amino acids + horse serum + albumen, the flukes developed to different extents at different times, causing difficulty in making valid

comparisons between media. Under 'good conditions' excellent results were obtained, masses of sperms in the seminal vesicle, extensive vitellaria and a large yolk reservoir being present. The sperm and vitellaria masses were easily visible in up to 90% of the flukes. Yet at another time examination revealed sperms in only about 50% and vitellaria in only 30% of the flukes. Two possible explanations are: (a) the differences in the serum and/or albumen in the medium may contribute partly to the variability in the level to which the flukes develop; (b) variation in the intrinsic capability of the metacercariae to develop may exist, i.e. morphologically similar larvae may not be in the same physiological condition. Although variation due either to serum or albumen could be eliminated when a successful medium containing only defined components is developed, it seems almost impossible to eliminate variation which might be due to the metacercariae themselves. Such variation strictly speaking would only be eliminated if metacercariae of identical genital constitution were used, this of course is impossible, but it would be worthwhile determining whether the age of the metacercariae, temperature at which the fish has been kept, and the nutritional condition of the fish in any way affects the ability of the metacercaria to mature in the final host.

Although metacercariae can mature to the extent of producing sperms, vitellaria and abnormal eggs in the BSSG + yeast extract + amino acids + horse serum + albumen medium, it was discovered that adult flukes which had completed their maturation *in vivo*, produced normal eggs for 24 hours but abnormal thereafter, when transferred to this medium. The failure of these flukes to continue to produce viable eggs in cultures suggests that their metabolic activities were not maintained at the 'normal' adult level; it is probable that the metabolism of the flukes during development *in vitro* is quite different from that *in vivo* and hence only abnormal eggs were produced in cultures. Two probable explanations are given to account for the apparent difference of metabolism *in vivo* and in cultures: (i) the culture medium is deficient in some dietary essentials; and/or (ii) the physico-chemical character of the medium is different from that of the host environment, and unsuitable for the normal development of the flukes. Probably in the case of *D. phoxini* both are true, but available evidence suggests that too much emphasis should not be placed on trying to identify specific 'growth factors' without frequently re-assessment of the physical conditions. For instance, Bell (1958) suggested that the failure of *D. phoxini* to form normal eggs in yolk

and albumen, an excellent growth medium, was due to the existence of oxidising conditions in the medium which caused the oxidase-protein complexes in the vitelline cells to become prematurely oxidised and consequently the egg-shell was unable to form normally. Similar observations were made by Hopkins (1952) while culturing Schistocephalus solidus in a high oxygen tension environment; however, when cultured in semi-anaerobic conditions, Schistocephalus produced normal eggs. It was discovered in the present work that the addition of alpha-tocopherol, a compound known to possess reducing properties, to BSSG + yeast extract + amino acids + horse serum + albumen medium not only increased the number of D. phoxini producing eggs but also improved the shape of some of the abnormal eggs in cultures. Although the beneficial effect of alpha-tocopherol in cultures could be due to the fact that this substance is an essential nutrient of the flukes, it is also likely that the incorporation of alpha-tocopherol in the medium assisted in lowering the oxidation potential. The above observations with Diplostomum and Schistocephalus demonstrates the importance of providing an environment of a low oxygen tension in cultures for intestinal helminths. Unfortunately it is not always possible to determine what is the optimum physical conditions, since this may depend on the medium.

This is well illustrated by the fact that Bell (1958) found a pH of 7.4 or more gave best development in yolk + albumen, a figure substantiated by Wilson (1960) who found development, which in yolk (pH 6.3-6.5) was poor, was very much better if the pH was raised over 7.0. Yet in the present work the best results were obtained in a pH range of 6.1-6.5.

It does not seem worthwhile to continue at the present the elucidation of the nutritional requirements of D. phoxini in vitro, since 'normal' metabolic activities of the worms have not been maintained in the culture media containing complex substances, which have so far promoted development of the worms. Since development is abnormal, it is possible that an essential substance may not be 'wanted' as metabolism does not get to that stage. Development using a purely synthetic medium does not seem very promising; although as described horse serum or yeast extract or albumen can be omitted and a synthetic supplement added which gives to a greater or less degree the same effect, when two, or even more when all, are omitted simultaneously the synthetic supplements, have little effect. One is forced to the conclusion, therefore, that it is too soon to try to use a chemically defined medium. The alternative is to use as others, for example, Mueller

(1959) for Spirometra mansonoides, Bernzten (1961) for Hymenolepis diminuta, have done so successfully in recent months, a complex medium, and concentrate on getting 'normal' development in vitro. These experiments will tell one little about the nutritional requirements of the worms but they will tell one a lot about the physical conditions required and the form in which the nutrient need be supplied, i.e. solid, viscous or liquid. Having determined these physical conditions first, and even allowing for the fact as mentioned above that the optimum condition may sometimes vary with the substrate, it seems to be a time would have been reached when the chemical requirement could once more be investigated.

In considering the advantages and disadvantages of building up a simply defined medium supporting little development or breaking down a complex very 'undefined' medium, the possibility should not be lost sight of that another approach altogether may be best. One such approach has been briefly mentioned and investigated, that of starting with worms which are nearly mature, i.e. requiring only a few hours to complete development, or even culturing worms which are mature. Originally this seemed difficult because adults occur in a septic environment, but the development of antibiotics in the last 15 years had made this a much less formidable

obstacle to *in vitro* cultivation than it was. As physico-chemical conditions are improved so progressively younger and younger stages could be used. In this way one may presume the initial problem would be merely one of providing a suitable physical background, namely osmotic pressure, pH and redox potential, and probably an energy substrate such as glucose. To maintain egg production for several days, however, would require lipoid and protein syntheses, but the enzyme systems would already be present, only the protein and lipoid precursors need be supplied. Once normal egg production had been maintained for 2-3 days, the use of early stages in which for instance only the male genital system was fully developed could be used and so step by step progress could be made back to the metacercaria starting point.

SUMMARY

1. The advantages of using *in vitro* cultivation as a method for investigating the nutritional requirements of intestinal helminths are described.

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

3. Autolysed yeast extract promoted considerable development of the genitalia of flukes when added to a medium consisting of glucose-saline + amino acids + albumen + horse serum.

4. A series of procedures were applied to yeast, horse serum and albumen respectively to determine the size and nature of their active components.

   (a) Yeast

   Two active fractions were found in yeast (i) a dialysable fraction, the effect of which can be replaced by amino acids, (ii) a non-dialysable fraction which is resistant to mild acid and alkaline hydrolysis and is basically charged. Part of the non-dialysable fraction appears to be a small molecular substance occurring in a bound form, since it is lost if dialysis is

preceded by mild acid hydrolysis. Supplementation of a basal medium containing gluco-saline + albumen + amino acids + horse serum with a B vitamin solution containing nine vitamins including pyridoxine improved development considerably though the results were poorer than those obtained with yeast. Vitamin B_6 alone gave as good results as the whole vitamin supplement.

(b) Horse serum

Two active fractions were found in horse serum; (i) a dialysable fraction and (ii) a non-dialysable fraction.

The active substances in the dialysate of serum also occur in yeast extract and are therefore assumed to be water soluble and thermostable. The activity of the dialysable fraction was not replaced by co-enzymes, nucleic acid derivatives or inorganic salts.

Part of the activity of the non-dialysable fraction appears to be associated with the globulin protein fraction of horse serum. Alpha-, beta- and gamma-globulins of horse serum, respectively, promoted development of the flukes

when used as supplements to a basal medium containing gluco-saline + amino acids + yeast extract + albumen.

(c) Albumen

Dialysis and enzymatic hydrolysis of albumen greatly altered its activity in culture media. Part of the activity of albumen can be replaced by amino acids. There was an increase in the amount of amino nitrogen in albumen on incubation at 40°C. There was also evidence that albumen produces detoxifying effects in media.

5. Liver extract was found to be an effective replacement for yeast extract in media. The activity of the liver extract increases on autoclaving.

6. Eleven-day chick embryo extract gave almost the same activity as horse serum in cultures. Although there was very little loss of activity of the extract on dialysis, the activity was almost lost on heating to 80°C for 10 minutes.

7. Alpha-tocopherol increased egg production when added to a medium consisting of gluco-saline, amino acids, yeast extract, albumen and horse serum.

8. Flukes which had commenced their final stages of

maturation *in vivo* before being transferred to cultures showed as much variation in the level to which they developed in culture as metacercariae taken straight from the minnow's brain.

9. Flukes which had completed their final stages of maturation *in vivo* produced normal eggs for 24 hours but abnormal thereafter when transferred to cultures.

10. Suggestions for future work in the *in vitro* cultivation of *D. phoxini* are discussed.

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STUDIES ON THE NUTRITIONAL REQUIREMENTS OF DIPLOSTOMUM PHOXINI (STRIGEIDA TREMATODA) IN VITRO

The elucidation of some of the nutritional requirements of the strigeid trematode, Diplostomum phoxini, developing to maturity in vitro was attempted.

D. phoxini can develop in a medium containing glucose balanced salt solution, amino acids, yeast extract, egg albumen and horse serum at 40°C. Sperm production in this medium was often as good as in flukes matured in vivo although only in about 5% were eggs formed, and these were abnormal. It was discovered that the addition of alpha-tocopherol to the medium not only increased the number of flukes producing eggs but also improved the shapes of eggs produced in cultures; some of these eggs although not viable possessed the shape of a 'normal' egg.

In order to determine the nutritional requirements of the flukes, a number of procedures were applied to each of the complex biological substances in the medium, namely yeast extract, horse serum and egg albumen, to discover (a) which fraction was active, (b) the nature of the active fraction and the extent to which a chemically defined supplement can replace it. Two active fractions were found in yeast, (i) a dialysable fraction, the effects of which were replaced by amino acids and (ii)

a non-dialysable fraction, which was resistant to mild acid and alkaline hydrolysis and was basically charged. Part of the non-dialysable fraction appeared to be a small molecular substance occurring in a bound form, since it was lost if dialysis was preceded by mild acid hydrolysis; this substance was found to be in part replaceable by pyridoxine or some form of vitamin B_6. Supplementation of a basal medium containing glucose balanced salt solution + albumen + amino acids + horse serum with a B vitamin solution containing nine vitamins including pyridoxine produced only about 50% of the level of development reached when the medium was supplemented with yeast extract; a better level of development was obtained if the basal medium was supplemented by a high concentration of vitamin B_6 alone.

Two active fractions were found in horse serum, (i) a dialysable fraction which is also present in yeast extract and (ii) a non-dialysable fraction. No suitably defined component was discovered as a substitute for the active dialysable substances of serum. Part of the activity of the non-dialysable fraction appears to be associated with the globulin protein fraction of serum. Alpha-, beta- and gamma-globulins of horse serum promoted development of the flukes when used as supplements to a

medium consisting of glucose balanced salt solution + yeast extract + amino acids + albumen.

Albumen was only partially replaced by a solution of amino acids in cultures. It was discovered that there was an increase of amino nitrogen produced by albumen in the medium on incubation at 40°C. This suggests that the growth promoting substances may not be preformed but be continuously supplied to the medium during incubation. Enzymatic hydrolysed albumen and peptic cleavage products of albumen were found to be ineffective substitutes for native albumen in cultures.

Aqueous extracts of two natural products other than those previously used, were found to be suitable medium constituents for the development of D. phoxini. Autoclaved extracts of liver prepared from the liver of several species of animals were found to be successful replacement for yeast extract in media; and eleven-day chick embryo extract gave almost the same activity as horse serum in cultures. In the presence of yeast extract in the medium most of the activity of embryo extract was, like serum, confi to its non-dialysable fraction. Heating embryo extract to 80°C for 10 minutes completely destroyed its activity.

When adult flukes which had completed their final

stages of maturation *in vivo* were transferred to a culture medium (gluco-saline + horse serum + amino acids + yeast extract + albumen) they produced normal eggs for only 24 hours and abnormal thereafter, demonstrating that the 'normal' adult metabolic activities of the flukes were not maintained. The possibility that the production of abnormal eggs is largely due to wrong physical conditions is discussed, and a sequence of experiments is described which might permit the establishment of correct physical conditions.

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