THE BIOCHEMISTRY AND PHYSIOLOGY OF DIGENETIC OVA AND LARVAE

by

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ABSTRACT

A survey of digene life cycles was undertaken, in order to find a species which would yield sufficient material for a study of egg physiology and biochemistry. Fasciola hepatica was chosen as an experimental animal and a broad but intensive study of the egg was carried out.

The general structure of the egg and miracidium was investigated by conventional histological techniques and special emphasis was placed on morphological features having relevance to the experimental work.

The structure of the shell and membranes was determined using electron microscopy and chromatographic techniques. The permeability of the shell and membrane system was also investigated by means of simple physical experiments, and by use of radio-tracers. An explanation of the function of the shell, and of the origins and function of the 'vitelline membrane complex' was put forward.

The nature and utilisation of food reserves during development was studied by histochemical means and by chemical analysis. The changes in dry weight and respiration of developing eggs were also measured and the underlying biochemistry was discussed.

The structure and function of the flame cell system was investigated by studying variations in flame cell activity, and by observing the effect of vital dyes. An interpretation of the function of the system was advanced, based on the experimental results obtained.

The hatching mechanism was studied in a variety of experiments,

and the chemical properties and fine structure of the viscous cushion were determined.

The hatching enzyme theory postulated by Rowan was seriously questioned, and an alternative hypothesis, taking into account the hydrodynamic properties of the viscous cushion, was put forward.

Finally, observations on the factors affecting miracidial behaviour were recorded and the overall biology of the egg and miracidium was discussed.

INTRODUCTION

It has become increasingly apparent over the years that control of parasitic infestations demands a detailed knowledge of all aspects of the life cycle, particularly those sections concerned with transmission of the parasite.

Research on the digene egg and miracidium has been confined to a few aspects only. In certain fields (for example, taxes of the miracidium) our knowledge is detailed, whilst in others (such as utilisation of food reserves during embryonation) it is almost non-existent.

Experimental studies have been mainly confined to Schistosoma and Fasciola, though many observations on other flukes have been reported (e.g. Barlow, 1925; Onorato and Stunkard, 1931; Campbell, 1961). The formation and structure of the egg shell have attracted several investigators, the two principal papers on this subject being one by Stephenson (1947) and the review by Smyth and Clegg (1959). Rowan (1962) has studied the permeability of the shell and vitelline membrane.

A histological picture of embryonation was given by Schubmann (1905) and Ortmann (1908) though some of their interpretations are doubtful. Recently the chemical constituents of the vitelline cells were described by Ranzoli (1956) using histochemical techniques. The QO_2 and glycogen utilisation of developing Fasciola eggs were determined by Horstmann (1962).

The bionomics of the egg and factors affecting hatching have attracted attention in view of the economic importance of the parasites.

Fasciola has been investigated principally by Rowan (1956, 1957) and Rowcliffe and Ollerenshaw (1960), and Schistosoma by Standen (1951) and Ito (1955).

The biology of the miracidium is known in somewhat greater detail, though reports are often contradictory, particularly concerning the existence of a chemotactic response to the intermediate snail host. Wright (1959) has briefly reviewed the subject.

The histology of the miracidium of Fasciola has been reviewed by Mattes (1949) and Dawes (1960a) and of Schistosoma by Ottolina (1957). Work on the ultrastructure is confined to papers on the eyespot, by Kümmel (1960) and Isseroff (1963, 1964), and on flame cells, by Kümmel (1958, 1959). Bogomolova (1957) has described the chemical composition of the miracidium of Fasciola using histochemical techniques.

The process of penetration into the snail has been studied in detail by Dawes (1959, 1960 b, c).

These papers are selected from a much larger body of material. The overall picture is, however, fragmentary and reports differ widely in the depth of their approach. It therefore appeared that a detailed study of the physiology and biochemistry of the digene egg would prove worthwhile.

In order to obtain suitable parasite material it was decided to investigate several digene life cycles and to select the most favourable as a basis for more detailed study. The results of the brief survey carried out are presented in Part I. The major aim of the present work was to investigate the morphology, physiology, and biochemistry of the egg

and miracidium of the fluke chosen for study. Subsequent parts therefore deal with the following aspects.

a) Embryonation and the general histology of the egg and miracidium.

b) The structure and permeability of the shell and vitelline membrane.

c) The nature and utilisation of food reserves during development.

d) The structure and function of the flame cell system.

e) The hatching mechanism.

f) The behaviour of the miracidium.

PART I. A SURVEY OF DIGENE LIFE CYCLES.

Introduction.

Several criteria were evaluated in making the choice of a species suitable for experimental purposes.

(a) Maintenance of Hosts in the Laboratory.

(i) Final hosts. The choice here is governed by facilities available for maintaining the vertebrate host. Fish, amphibia, and reptiles require extensive apparatus if they are to be kept in any numbers, and behavioural features such as hibernation may interfere with experimental work. There is also the difficulty of administering the parasite and collecting the faeces. Birds or mammals would therefore seem to be preferable.

(ii) Intermediate hosts. Maintenance of the freshwater snail host is not a great problem, but its reproductive cycle must be taken into account. Most British snails appear to produce only one generation per year, and this would entail obtaining a year's supply of snails each Spring. There are also several little-studied factors which may be of importance. A relationship between susceptibility to infection and age of snail has often been postulated, and recent work (Kendall and Ollerenshaw, 1963) has shown that growth periods of Fasciola, in Limnaea truncatula are closely related to growth of the host.

(b) Life Cycle of the Parasite.

Nimmo-Smith (1962) has stated "Availability of material is one of

the most important rate limiting factors in the biochemical study of parasites". If, therefore, a life cycle is excessively long or devious, then a limitation is imposed on the amount of material which can be produced. Most flukes probably complete only one cycle per year under natural conditions.

(c) Size of Parasite.

For obvious reasons the general rule is the greater the size the better, for experimental purposes. One of the main obstacles to progress in Parasitology is the diminutive size of the material.

It would seem that the number of digenes suitable for study is large. However, the choice was considerably reduced for several reasons, before commencing the survey. Schistosoma and Fasciola are the most commonly used mammalian flukes, but for a study of egg physiology, Schistosoma is particularly unsuitable. Embryonation occurs within the host tissue and there is no control over age, number, or contamination of eggs separated from faeces or urine. In spite of the work of Newsome (1962) on the maturation of schistosome eggs in vitro, the situation remains unsatisfactory. Fasciola, on the other hand, has attracted some attention and large quantities of clean eggs may be obtained from bile or mature flukes. Techniques for infecting the snail, and the final host are in frequent use in many laboratories.

Bird hosts would also seem to provide a ready source of parasites. Nicoll (1923) in his survey of bird trematodes, concluded that the

Anseriformes were most commonly infected.

A survey of anseriform digenes was therefore carried out, and the possibility of using Fasciola was investigated. The results were then evaluated with the above criteria in mind, and the most suitable parasite was chosen.

Methods.

In the Autumn of 1962 attempts were made to obtain Mallard from the few remaining duck decoys in East Anglia. The proprietors were, however, unable or unwilling to supply the birds. A small number of wild duck were obtained from other sources in the South of England, and the intestines were examined for flukes.

Collections of the commoner freshwater gastropods were also made in 1962-63. The area around Imperial College Field Station, underlain by Bagshot Beds, is unsuitable for molluscs. Collections further afield, mainly at Pevensey Levels (Sussex), Bushy Park (Middlesex) and the Basingstoke Canal (Surrey and Hampshire), were more successful.

The molluscs obtained were sorted into species and placed in deep, 3" diameter, glass dishes (2-4/dish). If cercariae were not detected, the snails were subsequently dissected to ascertain whether larval stages were present.

A large amount of material was obtained in this way, but much of it could not be identified with certainty. Feeding experiments were carried out with selected infective forms. These were administered in a variety

of ways, and details are given in the relevant text. Faeces were examined daily by sieving and sedimentation, and by floatation in 50% zinc sulphate. All other techniques tried were found to be unsatisfactory.

The breeding habits of a number of freshwater snails were investigated in the laboratory. The snails were maintained in dechlorinated tap water on a diet of artificial alginate (Standen, 1952) at a temperature of 17 - 20°C. The time required to reach maturity, number of eggs laid, and embryonation time were all noted and a comparison of rates of growth recorded. On hatching, the juvenile snails were placed in dishes containing dead sycamore leaves (Krull, 1937) and after sufficient growth had occurred they were transferred to alginate.

A culture of Limnaea truncatula was obtained from the Central Veterinary Laboratory, Weybridge, for comparison with the freshwater snails. Limnaea truncatula requires a special diet of the blue-green alga Oscillatoria sp. which can be grown on slopes of damp mud (Taylor and Mozley, 1948). The soil must have a high calcium, potassium, and phosphate content. Local soil proved ineffective, and earth fulfilling these requirements was obtained from Somerset.

Livers infected with Fasciola were obtained from slaughterhouses and flukes and eggs were removed from the bile ducts. Eggs were embryonated by the method of Jepps (1932) and snails about 2 mm long were exposed to 1 - 3 miracidia to determine whether infections could be easily and reproducibly obtained.

Results.

(a) Surveys.

Ten wild duck were obtained in October and November, 1962. Of these, three were infected with hymenolepid tapeworms, two with Capillaria sp., one had acute tuberculosis, and one was infected with echinostome flukes in the small intestine. Unfortunately, the flukes were in a poor state of preservation and could not be accurately identified. They may have been Echinoparyphium recurvatum. Attempts to infect snails were not made because the intermediate host was not known with any certainty.

Examination of freshwater snails was more successful and a proportion of those collected in any locality was almost always infected. Limnaea peregor was the most commonly parasitized of the species examined. Sporocysts shedding furcocercariae or xiphidiocercariae were found in specimens from all localities, whilst L. peregor from Bushy Park, Middlesex, were also shedding monostome and echinostome cercariae on different occasions. Limnaea stagnalis was found to be parasitized only by sporocysts shedding xiphidiocercariae, in all localities. L. palustris was found only on Pevensey Levels and was infected with large rediae, later correlated with a large echinostome metacercaria. The Planorbidae were, in general, less parasitized, though 1% of P. corneus from Pevensey Levels were infected with unidentified rediae. Of prosobranch molluscs encountered, Bithynia tentaculata occasionally harboured sporocysts shedding furco- and xiphidiocercariae whilst B. leachii and Vivipara fasciatus were not

parasitized.

Infective stages were of more immediate interest. Planorbids and Limnaeids from Pevensey were found in 100% of cases, to harbour the large echinostome cyst mentioned above, and later identified as Cercaria limbifera.

L. pereger, from Bushy Park, harboured numbers of a 43-spined echinostome metacercaria and P. corneus, a 37-spined echinostome metacercaria. Encysted monostome cercariae were also found at Bushy Park on L. pereger shells, vegetation, etc.

One of the species of furcocercariae infesting L. pereger in all localities was identified as Cercaria A and its metamorphosed form Tetracotyle typica was abundant in L. pereger in these localities. It is the larval form of the Strigeid fluke Cotylurus cornutus.

These five infective stages were selected for feeding experiments using ducks, pigeons, and chickens as hosts. Descriptions of parasites and experiments performed are given below.

Cercaria limbifera: the cysts were found mainly in the kidney of snails from Pevensey. They were very large (350 - 390 μ in diameter) with a wall 25 μ thick of two distinct layers (Fig. 1a). The cyst contained a juvenile worm with a prominent crown of 35 spines. These were arranged in two groups of four, one on each ventrolateral lappet of the collar, a row of eight on each side, and a double row of eleven lying dorsally (4 + 8 + 11 + 8 + 4) (Fig. 1b).

Two species of 35-spined echinostome cercariae are described in

Fig. 1. Digene Life Cycles.

a. Cyst of Cercaria limbifera.

b. Head of Cercaria limbifera showing arrangement of spines.

c. Echinostoma londonensis.

d. Head of E. londonensis showing spine arrangement.

e. Tetracotyle typica, metamorphosis.

f. " " "

g. " " infective cyst.

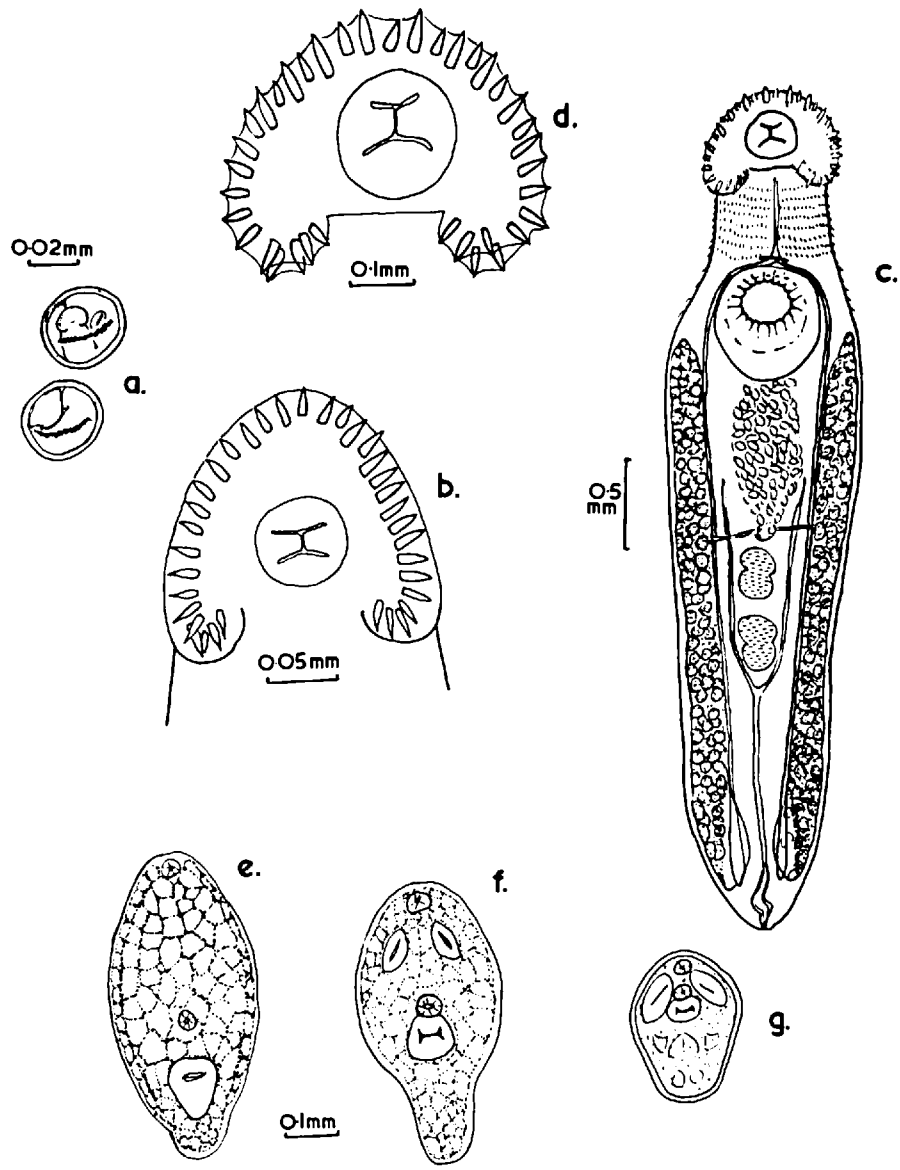


Fig.1 Digene Life Cycles

the literature (1) C. limbifera described by Seifert (1926) and redescribed by Brown (1931) and Rees (1932) in Britain, and (2) C. echinostomi described by Dubois (1929) and thought by Wesenberg-Lund (1934) to be identical with C. limbifera. The final host of C. limbifera is unknown, although Rees records that 82 cysts fed to a duckling did not produce an infection. She attempts to identify the metacercaria with Echinostomum aluid from Phoenicopterus ruber, the flamingo.

The cysts obtained were fed to Mallard, pigeons and chickens in a variety of ways: in whole snails; dissected out in gelatine capsules; and suspended in saline, using a Pasteur pipette. Daily faecal examinations were negative. The birds were killed 20 - 22 days after feeding of cysts and in no case were flukes recovered.

Tetracotyle typica: on the Continent the snail host of Cercaria A, and Tetracotyle typica is L. palustris. In Britain, however, it has been found in L. pereger by Harpur (1931) and little can be added to his description. The cercariae are produced in long filiform sporocysts and shed daily in thousands. They then penetrate a second snail and metamorphose into the infective Tetracotyle (Fig. 1e, f, g). In two cases, the tetracotyle larvae were dissected out from L. pereger and fed to ducklings to avoid a mixed infection with the echinostome metacercariae also present. In a third experiment whole snails were used, but in no instance were infections obtained.

37-Spined Echinostome: Khan (1961) described a new species of echinostome,

Echinostoma londonensis from material collected at Bushy Park. The fluke is identical in all respects with E. revolutum but the infection could only be demonstrated in pigeons. Zunker (1925) described E. columbae (which is also 37-spined) from pigeons and it is difficult to understand why Khan considered E. londonensis to be a new species. The 37-spined echinostomes found encysted in P. corneus were similar to Khan's material and it was concluded that they were E. londonensis.

Doses were administered to four pigeons, either as whole P. corneus or as metacercariae dissected out into 0.4% saline. Eggs were detected on the sixth day after feeding, in the two birds fed whole P. corneus. Autopsy revealed one fluke and six flukes respectively, in the lower small intestine (Fig. 1c, d).

An infection was not obtained in two ducklings fed three whole P. corneus each, which would tend to confirm Khan's findings on the specificity. No rediae were found in P. corneus and it was therefore presumed that the cercariae had been liberated the previous summer. It may be that their viability was somewhat reduced by overwintering in the snail.

43-Spined Echinostome: in the early Spring of 1963 L. peregeр from Bushy Park were found to contain large numbers of metacercariae of a 43-spined echinostome in the connective tissues. About 15% of the snails also harboured large yellow rediae which on a later occasion (July, 1963) were found to be shedding 43-spined echinostome cercariae. It was therefore concluded that the metacercariae discovered in April were probably shed the

previous summer.

Doses of 50 and 100 metacercariae were dissected out into 0.4% saline and administered to two ducklings by Pasteur pipette. One pigeon and one duckling received three whole L. peregor each. Eggs were detected in the faeces of the latter two birds on the sixth day after feeding. All four birds were killed 10 - 14 days after feeding and the flukes were recovered. The pigeon harboured 176 mature echinostomes in the upper small intestine, and the third duckling, 8 flukes in a similar location.

The question of identity was uncertain. Size of worms and their organs is not a good criterion for classification. Senger (1954) has shown that echinostomes continue growing after egg production has commenced. Hence, 'mature' flukes may vary considerably in size. The number and arrangement of spines are better criteria. The common Echinoparyphium recurvatum has 45 spines on the collar.

The intermediate hosts of this species seem numerous and Mönnig (1934) mentions five hosts. Mathias (1927) and Wesenberg-Lund (1934) both described it from P. planorbis and Dinulesco (1939) recorded it from Paludina vivipara. The latest description is that of Gmitter (1955) from L. pereger. Unfortunately, Gmitter's species had 45 spines also. It may be that we are dealing with a number of races, or with a species complex. It is recorded here, therefore, that the material from Bushy Park could be a new species. A short description is given below and this is illustrated in Fig. 2.

The rediae were pale yellow, with small locomotor appendages.

Fig. 2. *Echinoparyphium* species.

a. Mature worm.

b. Head to show spine arrangement.

c. Mature redia.

d. Cercaria.

e. Metacercariae encysted in snail connective tissue.

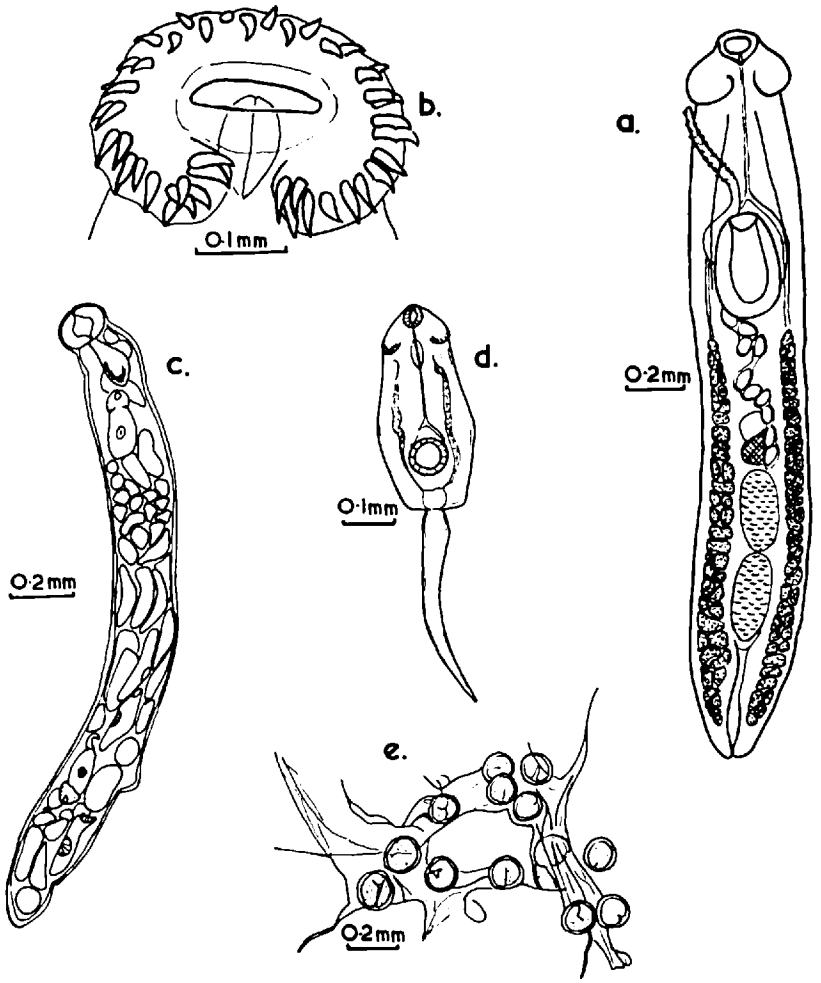


Fig. 2 Echinoparyphium sp.

The intestine was short, amounting to about 1/10 of the total length in mature forms. The cercariae were shed in the early morning. They swam actively for a short time before entering another snail, shedding their tails, and encysting.

The adult worms were rose coloured and the prominent crown of spines consisted of a group of five on each ventro-lateral lappet, with a double row of 33 round the collar between the two lappets. The surface of the neck region was covered with transverse rows of small spines and the size ratio of the ventral to oral sucker was about 3 : 1. The uterus was short, containing 8 - 10 eggs. These were whitish and thin-shelled with a small operculum. They embryonated in three weeks at 25°C. and hatched under the stimulus of light.

Dimensions (mm.)	Range	Mean
Eggs		0.06 × 0.1
Rediae	0.41 - 2.46 × 0.12 - 0.41	1.54 × 0.22
Cercaria body	0.27 - 0.39 × 0.096- 0.13	0.31 × 0.10
tail	0.31 - 0.41	0.36
10 day old adult	1.93 - 2.26 × 0.35 - 0.41	2.07 × 0.38
14 day old adult	2.87 - 3.4 × 0.49 - 0.67	3.1 × 0.57

Monostome metacercariae.

In April, 1963, approximately 15% of L. pereger at Bushy Park were shedding monostome cercariae. These encysted on vegetation etc., and particularly at the water-air interface of glass containers. The snails

were infected with rediae having long sinuous guts, but no locomotor appendages. The germinal masses were arranged in the characteristic monostome configuration, with only two to three developing cercariae present per redia. Many developing cercariae were found free in the tissues. They possessed well-developed eyespots and were strongly pigmented. The cysts were hemispherical, with the flat side adjacent to the substratum. Dimensions are given later and the forms described above are illustrated in Fig. 3.

Cysts were removed from glass dishes with a sharp razor blade and 1 - 200 were fed to each of three ducklings, using a Pasteur pipette. No eggs were detected in the faeces and the birds were killed 14 days after being fed cysts but no flukes were recovered. Two ducklings were then fed 1 - 200 cysts attached to vegetation etc. At autopsy, ten days after feeding cysts, four mature Notocotylus were found in the caeca of one bird.

The position and number of ventral glands are considered an important diagnostic feature in Notocotylus. In this case there were two lateral rows of 14 and a median row of 16 glands (Fig. 3b). The anterior gland of the lateral row was at a level about halfway between second and third median glands.

Dimensions (mm.)	Range	Mean
Rediae	0.78 - 1.23 × 0.14 - 0.21	0.96 × 0.17
Cercariae body	0.41 - 0.53 × 0.12 - 0.16	0.47 × 0.13
tail		0.57
Cyst diam.	0.19 - 0.22	0.21
wall	0.013- 0.016	0.015
Adult	1.72 - 1.90 × 0.45 - 0.53	1.82 × 0.49
Sucker diam.	0.11 - 0.12	0.12
Eggs		0.023× 0.013 (+ filaments)

Fig. 3. *Notocotylus attenuatus*.

a. Mature worm.

b. Mature worm showing ventral glands.

c. Redia.

d. Fully developed cercaria.

e. Surface view and cross section of metacercaria.

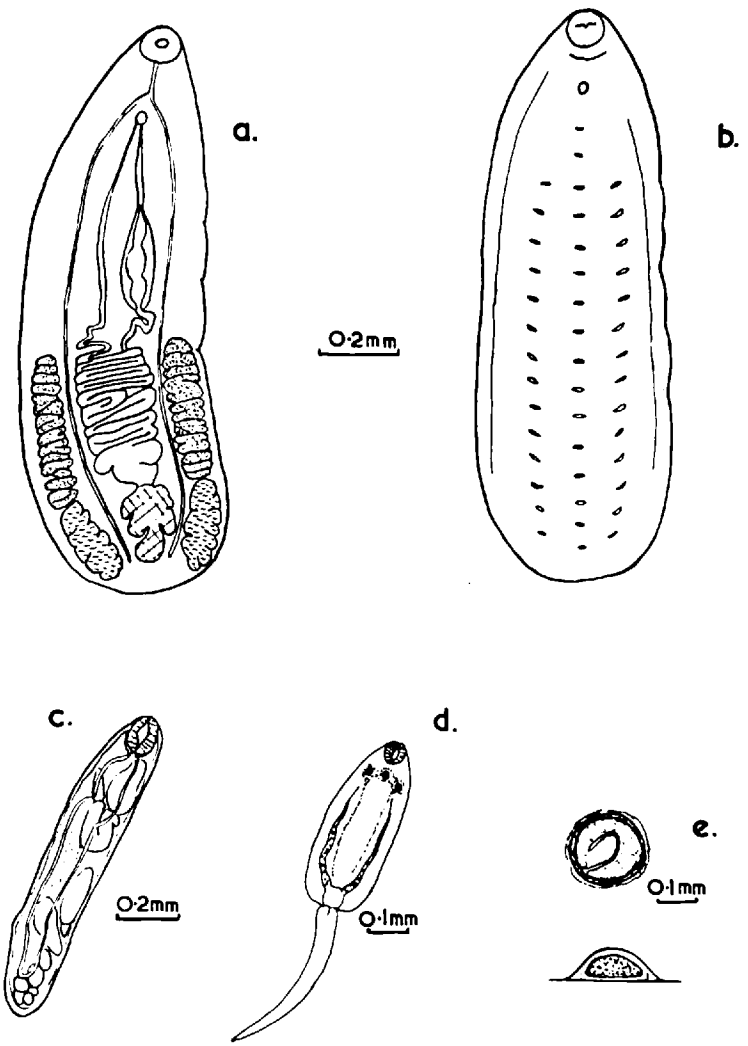


Fig.3 Notocotylus attenuatus

The genus Notocotylus is well documented. Dubois (1951) in his revision of the genus recognised 18 species of which four, N. ephemera, N. imbricatus, N. seineti and N. attenuatus, occur in Europe. Dubois reverts to the old name for N. attenuatus, i.e. N. triserialis. Examination of the ventral gland arrangement eliminates N. ephemera and N. seineti (Szidat and Szidat, 1933).

The cercaria of N. imbricatus, possessing two eyespots is a member of the urbanensis group of monostome cercariae, Cercaria vaga, the larval form of N. attenuatus, having three eyespots, belongs to the ephemera group (Szidat, 1935). The measurements and configuration of organs in the mature worm are similar to those given by Dubois for N. attenuatus. We must, therefore, conclude that this is the species under consideration.

(b) Breeding of Snails.

Stocks of freshwater snails were kept in large tanks of aerated water which was changed frequently. For breeding purposes it was found advantageous to isolate three to four snails in 9" diameter pyrex dishes containing about 1 inch of water. These did not require aeration and snails were transferred to fresh dishes when sufficient eggs had been laid. The snails, when maintained at 17°C., required about ten months to reach maturity. They would not commence egg laying below this temperature.

Though laboratory conditions and diet cannot be said to simulate exactly the natural environment of the snail, there is some justification for believing that freshwater snails complete only one life cycle per year.

Having laid their eggs they remain alive until the onset of winter and low water temperatures. Overwintering of these mature freshwater snails has not been achieved in the laboratory, though a single specimen of the terrestrial Helix pomatia has just completed a third period of overwintering.

Limnaea stagnalis and L. peregeri lay eggs prolifically under laboratory conditions. The eggs hatch in about twelve days at 17°C. Growth, however, is very slow, probably because nutritional requirements are not fulfilled. Planorbis corneus and P. planorbis lay fewer eggs than the limnaeids. The juvenile snails will eat only dead leaves, but growth is fairly rapid on that diet.

Limnaea truncatula seems to be an exception to these generalizations. It is amphibious in habit and considerably smaller than the other species. At 20°C. the eggs require about ten days to embryonate and the snails take only four weeks to reach maturity. Eggs are laid prolifically for a further six to ten weeks and the snails then die. Under natural conditions population build up must be fairly rapid and it has been estimated that in Britain at least two life cycles are completed each year.

(c) Fasciola.

No difficulty was encountered in embryonating the fluke eggs, and distilled water was normally used to keep microbial contamination to a minimum. If the water was not renewed from time to time it became markedly alkaline, moving from the pH 5 - 6 of distilled water to a pH of 8. Hatching was inhibited under these conditions though low oxygen tension may have been

a contributory factor. A change of aqueous medium would always result in 80 - 90% hatch. Oxygen is a necessary factor in development. It was found that cultures in 3 × 1 inch specimen tubes rapidly became stagnant and 50 ml. conical flasks were therefore adopted as incubation vessels. These could easily be covered with black paper to exclude light and prevent hatching of embryonated eggs.

Snails were exposed to miracidia in solid watch glasses or 3/8" diameter test tubes. In all eight experiments performed, infections were obtained in 20 - 55% of the snails. A minimum of twenty snails made up each experimental batch and ten day old snails gave slightly better results than other ages.

Discussion.

The lack of information on trematode ecology soon became apparent. Work has, in general, centred around descriptions of parasites found and little attention has been paid to the population dynamics of the host-parasite relationship. Significant contributions to our knowledge could be made in that direction, but the orientation of this work did not allow these lines to be pursued.

The purpose of the surveys was to facilitate the selection of a fluke with a suitable life cycle as a basis for experimental work. The results were accordingly assessed and a choice made.

The survey of birds for mature flukes was markedly unsuccessful. The inherent drawback here is that even if a fluke is identified the inter-

mediate host may be unknown or controversial. Collection of intermediate hosts is a better proposition and, whilst the identification problem still exists, feeding experiments are relatively easy to perform. Most life cycles were discovered in this way, but few workers have tried to complete the cycle by infecting the snail.

The results of feeding experiments were disappointing, and it is evident that more needs to be discovered about the conditions under which an infection can be established. Senger (1954) states that he stored metacercariae in 0.4% saline prior to use in experiments. However, it proved possible to establish infections here, only with cysts which had not been removed from snails or substratum (i.e. by feeding whole snails). The failure of other feeding experiments is not understood as cysts were in saline for only a few minutes.

The large cyst of C. limbifera would have been very suitable for study. The hosts used in feeding experiments were, however, probably not natural ones. Dietz (1909) mentions "Echinostoma sp." from the coot Fulica atra. Pevensey Levels provides a suitable habitat for these birds and they may constitute the true host.

In the case of Tetracotyle typica, the causes of failure of the feeding experiments are not known. Conditions and host would seem to be favourable.

The choice of material was therefore limited to Echinostoma londonensis, Echinoparyphium sp., Notocotylus attenuatus and Fasciola hepatica. Feeding experiments with the first three gave erratic results,

and only in the case of Fasciola could snail hosts be infected with certainty if the need arose. The snail hosts themselves would only breed once per year, except in the case of L. truncatula which would complete its life cycle in six weeks. The eggs of Echinoparyphium sp. would have been ideal for a study of cleavage and the germ cell cycle. The ovum was not obscured by a dark shell and yolk material, as in Fasciola. The eggs of Notocotylus are interesting in that they do not hatch until ingested by snails. They were, however, judged to be too small, and in all three bird species eggs were not produced in the tens of thousands necessary for experimental work. They were also difficult to isolate in a viable condition from faecal material.

Fasciola on the other hand could be obtained fairly regularly from slaughterhouses and provided many thousands of clean eggs. From the practical point of view Fasciola is of economic importance and any discoveries might have a useful application.

One further deciding point concerned maintenance of parasites. The time spent in tending the final and intermediate hosts in this survey, and obtaining infections, amounted to 50% of total research time. On the other hand a single day spent in dissecting fluky livers would provide sufficient material for several weeks of uninterrupted experimentation. Fasciola hepatica was therefore chosen as the most suitable source of experimental material.

PART II. THE MORPHOLOGY AND DEVELOPMENT OF THE EGG AND MIRACIDIUM OF FASCIOLA HEPATICA.

Introduction.

In the eggs of most Platyhelminths, food supplies for the developing embryo are stored in specialised vitelline cells. These are produced by numerous follicles or vitellaria which are thought to be transformed ovaries. The egg consists of a varying number of vitelline cells and a single ovum, enclosed within a tanned protein shell. Hyman (1951) considers that this situation has developed within the Phylum and is therefore a unique feature. Acoel and polyclad Turbellaria have normal endolecithal eggs. Most Allocoela show a transitional state with some oocytes becoming ova, and others forming follicle cells which surround and nourish each ovum. In the next stage of evolution the ovary differentiates into yolk and egg producing regions, and these finally separate, the yolk producing areas being called vitellaria. This last arrangement is found in trematodes.

Descriptions of digene eggs are usually relegated to a short paragraph in papers dealing with life cycles, and the embryonation is discussed only briefly. The embryology of the Fasciola egg has been described by Schubmann (1905) and Ortmann (1908). They interpreted the development in terms of the knowledge at that time, and their descriptions are not detailed. The main difficulty in observing the Fasciola egg is that the dark shell and abundant yolk almost obscure the developing ovum.

This is not the case in all trematodes and Rees (1940) has described the embryonation of an echinostome, Parorchis acanthus. Other accounts are given by Ishii (1934) for Fasciolopsis buski and Chen (1937) for Paragonimus. All agree fairly closely as to the course which development takes, though there are species differences. The ovum divides into a propagatory and a somatic cell. The propagatory cell ultimately produces the germinal cells, whilst the somatic cell divides many times to form the body of the miracidium. In addition, several smaller cells are produced which pass to the periphery of the egg and form the vitelline membrane. This last part of the account is enigmatic, as will become apparent later (Part III).

The miracidium of Fasciola has attracted more attention, but its minute size (120 × 20 μ) does not facilitate investigation of structure. It is not therefore surprising if many workers disagree in their interpretations. The first descriptions were those of Leukart (1882), Thomas (1883) and Coe (1895). More recent reviews are those of Mattes (1949) and Dawes (1960a). Miracidia of other species have been described in detail by Price (1931), Lynch (1933), Rees (1940), Ottolina (1957) and many others.

Our knowledge of miracidial structure is still incomplete and a satisfactory approach probably lies in the use of electron microscopy. Work in this direction has been carried out by Kümmel (1958, 1959, 1960) on the eyespot and flame cells of Fasciola, and by Isseroff (1963, 1964) on the eyespot of a philophthalmid miracidium. An extension of these

techniques to such features as the primitive gut, supposed sense organs, etc., would prove advantageous.

The structure of the egg and miracidium were investigated with two aims in mind: firstly to become familiar with the material and, secondly, to obtain information on points pertaining to the proposed physiological and biochemical work. Morphological features to which particular attention was given, and the function with which they are associated, were as follows:

(a) The shell and vitelline membranes and their role as a protective barrier against the external environment.

(b) Epidermal cells, germinal cells, and syncytial tissue, and their principal role in metabolism.

(c) Flame cells, ducts and associated structures, and their role in excretion.

(d) The viscous cushion and sacs ("oily masses" of Barlow, 1925) and their role in the hatching mechanism.

(e) The sensory system, primitive gut, and gland cells and their role in the behaviour of the miracidium.

These five main points provide a background to the experimental work and it was thus hoped to obtain a broad but detailed knowledge of the morphology of the liver fluke egg and miracidium.

Methods.

(a) Observations on living material.

Both developing eggs and miracidia were observed alive using

transmitted light and phase contrast microscopy. Miracidia are very active organisms and were restrained by using small pieces of lens tissue (10 × 1 mm.) placed beneath the coverslip. The miracidia very rapidly became moribund under these conditions and preparations were discarded after 2 - 5 minutes. (Any form of restraint upon the miracidium rapidly produces cytolysis).

(b) Histology of the egg.

Eggs are notoriously unsuited to histological practice. The presence of dense food reserves and toughened egg shell makes the material brittle, and sectioning is difficult. A further problem was encountered in Fasciola eggs. Here the permeability of shell and membranes is such that whilst water can rapidly leave the egg, alcohol takes time to penetrate. The result is a sudden and violent plasmolysis each time eggs are transferred to a higher alcohol concentration, so that by the time the clearing agent is reached, the egg contents are effectively homogenised.

This latter difficulty may be overcome by the use of aqueous, slowly penetrating, fixatives such as 1% osmic acid, or 5% formalin, and dehydration may be facilitated by making a small puncture in the shell and vitelline membrane, after fixation and prior to treatment with alcohols. Single eggs were cleared in xylene, embedded in paraffin wax, or by the ester wax method of Steedman (1947) and sections were cut at 2 μ thickness.

Squashes of developing eggs were also made, but did not produce good results, particularly in the later stages of development when the

embryo is easily damaged. Squashes were, however, successfully used in a histochemical study of undeveloped eggs which is described in Part IV.

Sections of eggs fixed in osmic acid were intensified using 1% Ethyl Gallate (Wigglesworth, 1959) whilst sections of formalin-fixed eggs were stained with Haemalum-Eosin (Pantin, 1960).

(c) Histology of the miracidium.

Whole mounts were obtained by smearing slides with albumen and pipetting a suspension of miracidia on to the slide. This was allowed to become tacky and fixed in Schaudinn's fixative, in the same way as one would treat a ciliated protozoan.

In order to obtain sections, miracidia were fixed in Carnoy's or Bouin's fixative, dehydrated, embedded in paraffin wax and sectioned at 2 μ. This proved tedious and a method was devised for obtaining sections of many miracidia simultaneously. A suspension of fixed miracidia was transferred to a capsule containing 5% gelatine solution. The solution was allowed to set and the block was trimmed, dehydrated, embedded, and sectioned, thus removing the manipulative difficulties encountered with a single miracidium or with free suspensions.

Sections and whole mounts of miracidia were used to investigate several features. The general histology was examined using Haemalum-Eosin, and the Feulgen technique (Feulgen and Rossenbeck, 1924). The sensory and nervous system was investigated using the silver impregnation technique of Bodian (1937) and an intra-vitam leuco methylene blue procedure (Pantin, 1960). Attempts were made to stain the excretory system using the method of Coutelen (1931).

(d) Histology of the viscous cushion.

Sections of developed eggs were poor and a histological examination of the viscous cushion (mucoid plug) was carried out, after its release from the egg. Unhatched eggs, suspended in a drop of water, or 2.5% gelatine solution, were pipetted on to a slide, and allowed to hatch. The slide was then dried and fixed in Schaudinn's fixative or Baker's Formalde-hyde-Calcium. The most reliable method was the dry smear technique as the gelatine tended to produce artifacts. The gelatine supported preparations were however the least distorted and cushions were easy to locate.

(e) Staining of ultra-thin sections.

The histological work revealed the limitations of the techniques immediately. Most of the interesting miracidial features are of small dimensions and the sections obtained were not adequate in revealing the necessary detail. Fortunately, at a later date the opportunity occurred of obtaining ultra-thin sections for light microscopy, stained by a toluidine blue technique. The technique is described later in Part III but the results are included here because the information obtained has most relevance in this section.

Results.

(a) Embryonation.

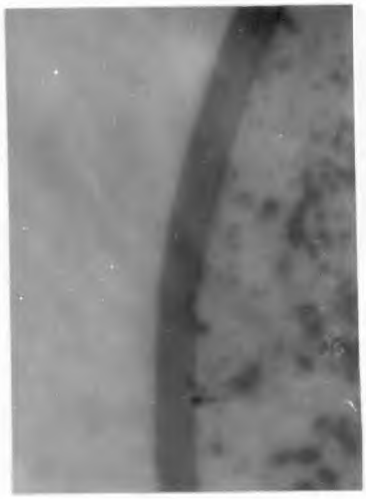
The undeveloped egg released from the fluke is enclosed in a light brown, tanned protein shell. Thin sections, stained by the toluidine

blue technique, have revealed that lying immediately beneath the shell is a layer of material about 0.2 - 0.4 μ in thickness, and of unknown composition (Plate 1a). This layer is considerably thicker in the fully developed egg (0.5 - 0.7 μ) (Plate 1b) and may possibly have been augmented by the "Hüllzelle" described by Schubmann and Ortmann, or the "vitelline membrane" described by Rees (1940).

The shell is 1 - 2 μ thick and appears homogeneous throughout. At one pole lies the operculum, demarcated from the rest of the shell by a thin opercular ring of unknown structure. The shell is thinnest in the opercular region, conceivably as a result of the moulding action of the uterus during shell formation. When the egg is first formed the ovum lies at or near the opercular pole but subsequently moves to a more central position. It is surrounded by twenty to thirty nucleated vitelline cells packed with food reserves (Fig. 4a and Plate 1c).

At 25°C. embryonation takes approximately eleven days. The single ovum rapidly divides to form a ball of cells, growing at the expense of the vitelline cells (Fig. 4b). In Fasciola it is not possible to observe this phase of development clearly because of the density of yolk and shell. Repeated attempts were made to find the vitelline membrane cells but these were not located. The vitelline cells retain their integrity but utilisation of food reserves does not proceed at the same rate in all cells. Some thus become rapidly denuded and vacuolated whilst others appear intact (Fig. 4c).

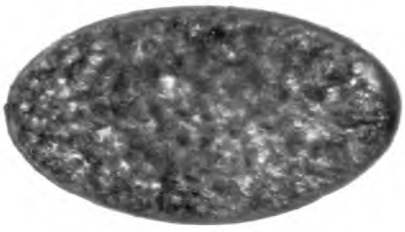
Cell differentiation commences about the sixth day of embryonation



2 μ

Plate 1a. Section of undeveloped egg, shell and vitelline membrane.

Plate 1b. Section of developed egg, shell and vitelline membrane.



25 μ

Plate 1c. Undeveloped egg.

Fig. 4. Embryonation.

a. Undeveloped egg.

b. Egg incubated for 2 days at 25°C. showing overall increase in vitelline lipid.

c. Egg incubated for 7 days at 25°C. showing utilisation of vitelline reserves.

d. Egg incubated for 9 days at 25°C. showing formation of the viscous cushion.

e. Fully developed egg.

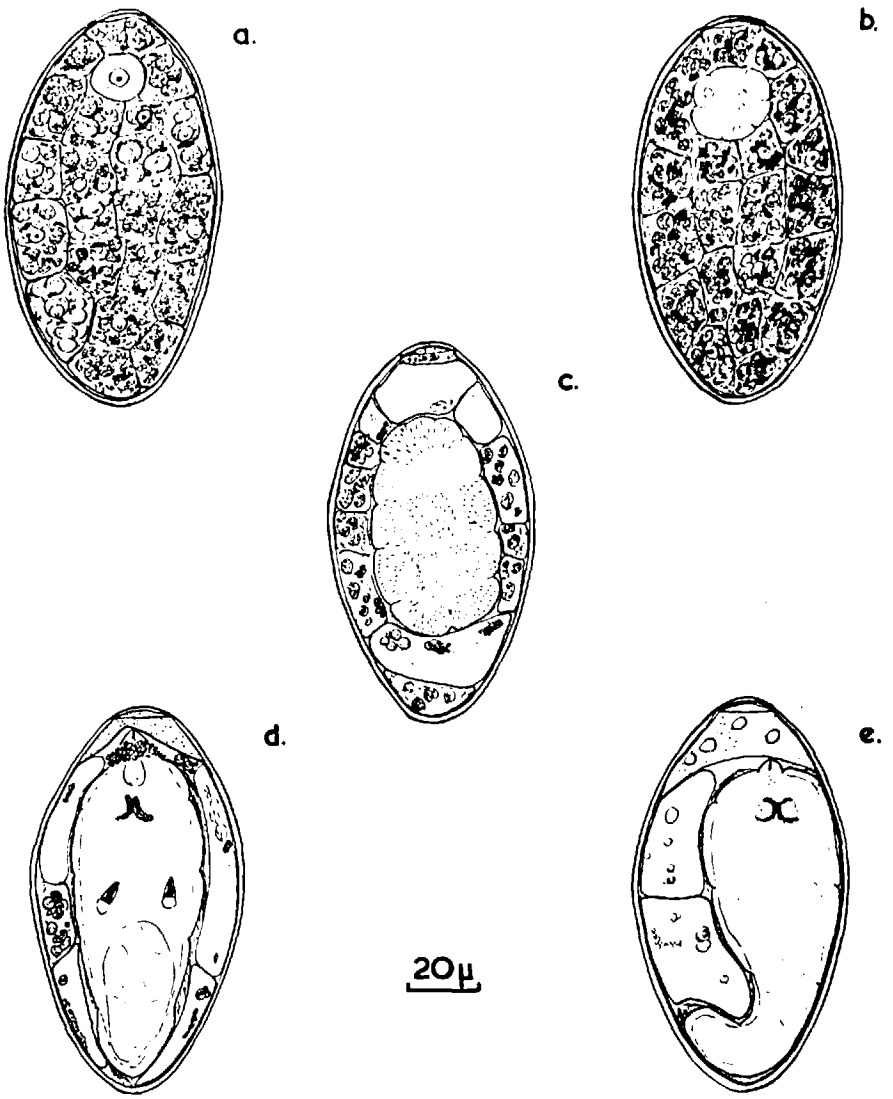


Fig. 4 Embryonation

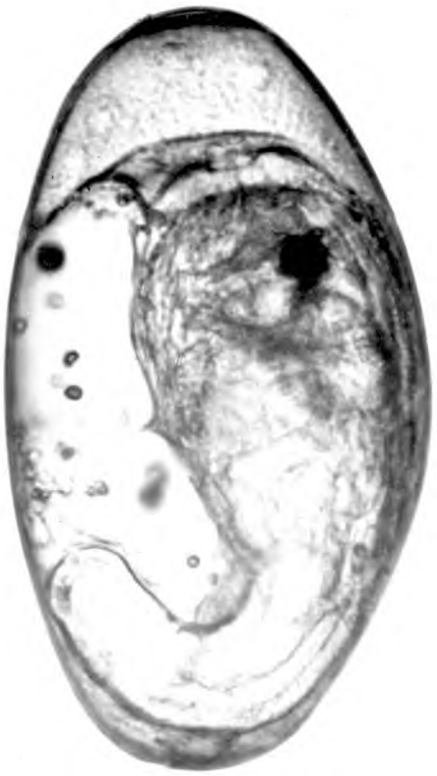
and by the seventh day the ciliated epidermal cells are clearly visible (Plate 2a). On the eighth day of embryonation the characteristic outline of the miracidium is recognisable and flame cells have developed. On the ninth day of embryonation, the eyespots and primitive gut are distinguishable and at about this time the viscous cushion appears beneath the operculum (Plate 2b and Fig. 4d). The germinal cells can also be seen in the posterior region of the embryo. By the eleventh day only two to five vitelline cells remain and these are equivalent to the sacs or "oily masses" of Barlow (1925). The fully developed egg is illustrated in Plate 3 and Fig. 4e. The flame cells are prominent but their activity is low. The primitive gut is filled with small granules and the three components of the eyespot are all visible (i.e. pigment cup, 'lens', and sensory region). The viscous cushion occupies about one sixth of the total egg volume and its histology is described below.

The mechanism whereby the food reserves are broken down and utilised is unknown, but many granules, possibly mitochondria or lysosomes are present in the cells and could play a prominent role. This particular aspect was not investigated in the later work and therefore remains a point of interest. The course of utilisation is, however, easily discernible. The cells first appear vacuolated and later completely denuded of material apart from a few small granules 1 - 2 μ in diameter which remain in suspension. The toluidine blue method indicates that these are possibly mitochondria.

A proportion (5 - 20%) of the eggs did not develop and some were

Plate 2a. Egg incubated for 7 days.

Plate 2b. Appearance of viscous cushion (Day 9).



20 μ

Plate 3. The fully developed egg.

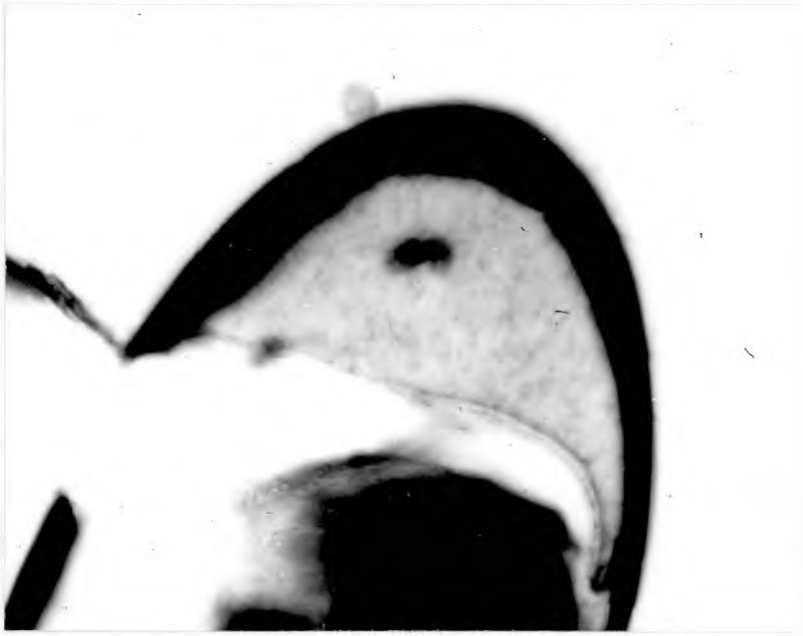
found to harbour a variety of fungal parasites. These are described in appendix I.

(b) Histology of the viscous cushion.

As mentioned above, the cushion appears at about the ninth day of embryonation as a small asymmetrical mass beneath the operculum. Plasmolysis of the egg in 1% saline caused the vitelline membrane to separate from the shell, showing that the cushion was surrounded by it. By the eleventh day the cushion is fully formed and large granules usually four in number, but varying from one to six, can be discerned in the matrix. The fully developed cushion appears to be delineated from the rest of the egg contents by a membrane. A few eggs were observed possessing two cushions, one at each end, and in these cases the granules were present in both, but in smaller numbers.

Rowan (1956, 1957) considered the cushion to be homogeneous throughout but observation on living unhatched eggs at × 1000 magnification revealed fine strands running through the matrix, especially in the vicinity of the large granules. This gives the whole cushion a finely reticulated appearance. Small granules were also seen but, being at the extreme limits of resolution, they may merely represent the junction of two or more fibrils. Fixation of eggs in osmic acid, A.F.A., etc., intensified the reticulated pattern and in sections the large granules appeared to be suspended in the cushion matrix by the fibrils (Plate 4).

Dry and gelatine supported preparations of hatched eggs stained



10 μ

Plate 4. Section of osmium stained egg showing fibrillar nature of cushion.

with nuclear stains, such as Mayer's Haemalum, revealed that the large granules were probably nuclei. Basic counterstains such as Neutral Red, Celestin Blue, and Pyronin Y all stained the ground substance of the cushion intensely, indicating that it was of a predominantly acid nature.

(c) Hatching.

Little can be added at this superficial level to Rowan's account of the sequence of events.

The miracidium is stimulated to hatch by exposure to light. The flame cell activity then increases rapidly and the miracidium becomes visibly activated. After a few minutes the cushion expands to approximately twice its original volume, compressing the miracidium and sacs. After a short interval the operculum ruptures violently, flies back, and the cushion streams out. This is followed by the miracidium which after a pause swims rapidly away.

(d) Histology of the miracidium.

Miracidial structure has been described many times and the general morphology is well known. It presents a remarkable example of complexity and miniaturisation. As mentioned earlier, Mattes (1949) and Dawes (1960) have given good accounts but both were concerned only with those organs of the miracidium involved in penetration of the snail.

The miracidium is covered by a layer of ciliated epidermal cells, apart from a proboscis-like region at the anterior end, termed a papilla or terebratorium. A region of syncytial tissue lies beneath the epidermal

cells. The posterior half of the miracidium encloses a cavity containing the large germinal cells. The prominent neural mass is situated in the mid-anterior region and above it are found the paired eyespots. The flask-shaped primitive gut lies in front of the neural mass and is flanked by small penetration glands. The excretory system consists of a pair of flame cells lying behind the nervous mass. From both cells, ducts pass laterally to open at excretory pores situated near the posterior extremity. This basic structure seems to be validated by most accounts but the presence of various other papillae, adhesive glands, etc., is not so firmly established.

i. Epidermal cells, subepidermal tissue and germinal cells.

The epidermal cells are arranged in a characteristic pattern. In Fasciola this is in the form of five tiers of cells and the number in each tier from anterior to posterior is 6, 6, 3, 4 and 2. The cells are separated from each other by cytoplasmic intrusions of about 1 μ width. The nuclei are of an irregular filamentous shape and the surface is granulated in both living and fixed material. The cilia are arranged in longitudinal rows and when active display a metachronal rhythm from the anterior to the posterior of each cell, implying a control mechanism orientated in that direction. The basal bodies were visualised using a variety of silver impregnation techniques, and found to be extremely small, having a diameter equal to that of the cilium. The whole of the epidermal cell beneath the insertions of the cilia is packed with round and oval mitochondria. This implies, as one would expect, an active oxidative metabolism

in this region.

In thin sections, longitudinal muscle fibres were revealed directly beneath the epidermal cells. Beneath these, the 'syncytium' can be divided into two regions. The outer of these consisted of discrete cells with large nuclei and clear cytoplasm. These cells were particularly evident around the germinal cavity. The central portion of the miracidium consisted of the syncytium proper, in which the brain, flame cells and other organelles are embedded. This tissue is rich in mitochondria, lipid droplets, granules, and minute tubules $\frac{1}{2} \times 3 - 4\ \mu$ in size. The nuclei of this region were small.

The germ cells are the largest cells of the miracidium and have dense granular cytoplasm rich in RNA but apparently containing mitochondria. The nuclei are very large with a prominent nucleolus.

ii. Excretory system.

A few points of interest emerged during the investigation and are described below.

In the free-swimming miracidium the flame cells lie horizontally with the nucleus towards the posterior extremity. The wide portion of the duct containing the cilia runs forward from the nucleus, narrows and turns downwards. It passes ventrally beneath the flame cell towards the posterior of the miracidium along a lateral wedge of cytoplasm. The ducts follow a tortuous course and are extremely difficult to observe, even in living specimens. It was not possible to ascertain whether they were intra-

or extracellular, but nuclei appeared to be associated with them in squashes.

The excretory pores lie one on each side between the fourth tier epidermal cells and near to their posterior borders. The last three to four microns of each duct is surrounded by a dense granular mass of cytoplasm which stains deeply with Neutral Red, and does not appear to have been described before. The duct opens into an 'atrium' some 2 microns in diameter before reaching the exterior at a pore 1 μ in diameter.

A large discrete triangular cell lies behind each excretory pore and may be distinguished from the germinal cells by its shape, smaller nucleus, and affinity for Methylene Blue and other basic dyes. There is also a pair of cells with similar staining affinity lying one on each side of the anterior border of the germinal cavity.

The former pair of cells were figured by Coe and Mattes. Lynch described somewhat similar cells in the miracidium of Heronimus. In their characteristic staining reaction they are very similar to the athrocytes of Turbellaria which are thought to have an excretory function. Lynch therefore termed them accessory excretory cells.

The method of Coutelen for flame cells and associated ducts was unsuccessful in both sections and whole mounts. The method is known to give excellent results with Cestode tissues (Wilson, 1962) and failure to stain the miracidium implies different structural components and possibly different functions in the two organisms.

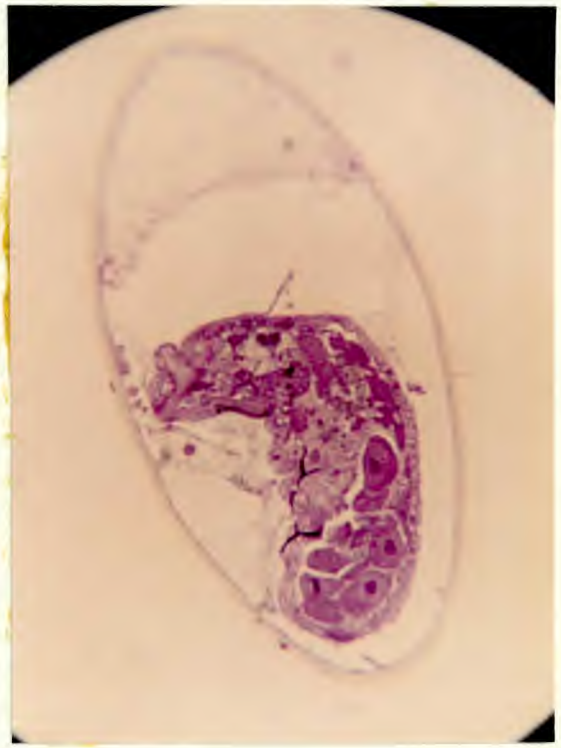
iii. Nervous system, primitive gut, and gland cells.

The nervous centre is situated directly beneath the eyespots and in thin sections appeared as a tangled mass of minute fibrils, surrounded by small nuclei. Silver impregnation techniques consistently failed to stain up this tissue or any nerves leading from it.

Each eyespot consists of a pigment cup of 'melanin' granules, a sensory region, and a bifid 'lens' which Kümmel showed to be packed with mitochondria (Plate 5).

A variety of papillae are featured by many workers and the most prominent of these is a pair lying laterally between the first and second tier cells. Dawes (1960) concluded that they assist early entry into the snail by giving purchase on the snail's epithelium. The thin sections revealed that the papillae do in fact have a rod-like core (1 × 3 μ) extending diagonally into the miracidium and they could well assist penetration into the snail. Immediately anterior to each lateral papilla lies a minute projection of cytoplasm which is hard to distinguish amongst the cilia of first tier cells. It has been described in the miracidium of Schistosomatium douthitti by Price (1932) and Schistosoma mansoni by Ottolina (1957) who termed it a satellite bristle. It was noted in the miracidium of Fasciola by Coe and more recently by Mattes.

The primitive gut consists of a granular syncytium containing three to four nuclei. A duct leads up the centre of the anterior papilla to open at its tip. It has been observed that miracidia which are trapped in lens tissue sometimes pass out the granules from the gut via this duct.



20 μ

Plate 5. Thin section of fully developed egg stained by the Toluidine blue technique.

The staining reaction of the granules is basophilic and this would imply a mucin component. The granules may in fact be analogous to the lysosomes of vertebrate tissue in containing digestive enzymes and thus aiding in penetration of the snail.

Lying laterally, one on each side of the primitive gut, are the accessory gland cells. In Fasciola they are minute and difficult to distinguish from surrounding tissue. Sections of the papilla revealed ducts leading from them to open on the anterior lateral region. Glands of this type are figured by Rees, Ottolina, Price, etc. in a variety of miracidia and are usually much larger, particularly in schistosomes. They are referred to as penetration glands but their small size in Fasciola may imply a reduced significance.

A composite diagram of the structure of the miracidium was reconstructed from the many observations and this is illustrated in Fig. 5.

Discussion.

The liver fluke egg is essentially of the freshwater type and one would therefore expect to find the range of modifications characteristic of these forms. However, as Hyman pointed out, the platyhelminth egg is also of a unique type and some variations from the common pattern are to be expected.

One of the main features of a freshwater egg is the presence of a barrier between contents and external environment to prevent dissipation of nutrients. The barrier is provided by the shell and vitelline membrane,

Fig. 5. The Miracidium.

Abbreviations:- a.e.c. accessory excretory cell; a. gl. accessory gland cell; a.p. anterior papilla; c. cilia; d. duct of primitive gut; e. epidermal cell; e.c.n. epidermal cell nucleus; e.p. excretory pore; f.c. flame cell; g.c. germinal cell; g.r. granular region; l. lens; l.b. lateral bristle; l.p. lateral papilla; m. mitochondria; n. neural mass; p.c. pigment cup; p.g. primitive gut; s.r. sensory region; s.e.c. subepidermal cell; s.e. syn. subepidermal syncytium.

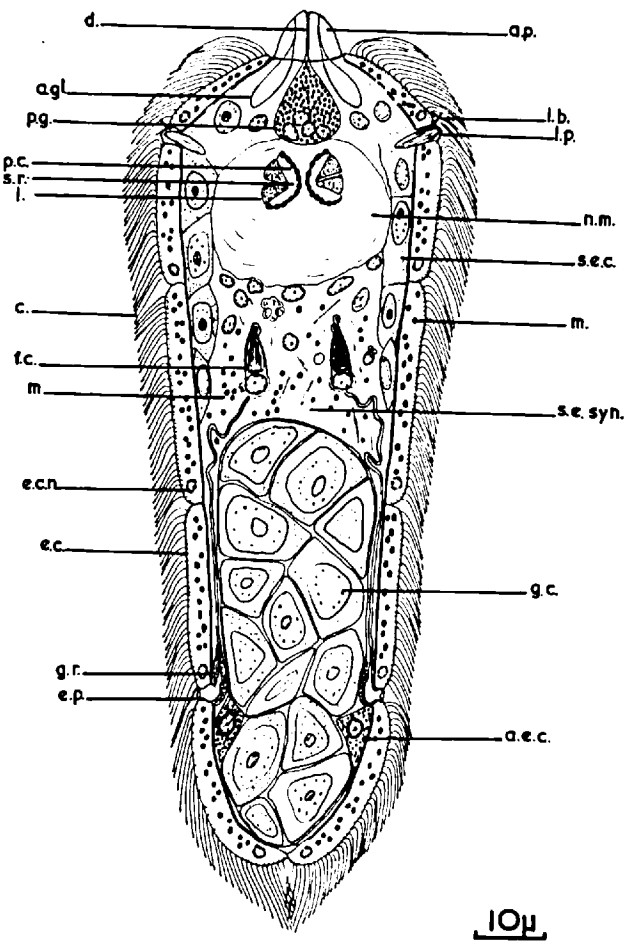


Fig. 5 The Miracidium

and in Fasciola their exact role requires some clarification. The concensus of opinion is that the vitelline membrane does not appear until midway through development but the barrier to outward diffusion must be present in the undeveloped egg.

The shell appears homogeneous throughout, apart from the opercular ring. The comprehensive accounts of shell formation by Stephenson (1947) and Smyth and Clegg (1959) appear authentic, and it is difficult to visualise how a discontinuity such as the opercular ring could arise in such a system.

The metabolism of the developing embryo has been the subject of two papers only. Ranzoli (1956) considered that carbohydrate was the principal constituent of the vitelline cell food reserves, and Horstmann (1962) also took this view. The situation cannot be dismissed so easily as lipid droplets could be discerned within the egg, particularly during development. The presence of mitochondria in the vitelline cells is also indicated. It would appear that these cells possess a full compliment of metabolic functions and are not simply bags of food. The utilisation proceeds in an orderly manner and this implies some form of control, not necessarily active, between the embryo and vitelline cells.

The epidermal cells and subepidermal syncytium of the miracidium also contain mitochondria. Their distribution is paralleled by that of glycogen (Bogomolova, 1957) and they probably represent the chief areas of oxidative metabolism in the miracidium. The significance of the cellular subepidermal layer is not understood. This layer consists of undifferent-

iated cells with large nuclei, which later come to form the outer wall of the sporocyst and may therefore play no immediate role in the miracidium.

The flame cell system, on little evidence, has often been credited with functions of osmoregulation and excretion. Flame cells are widespread throughout the lower invertebrates but a critical account of their function has not been published. The presence of a granular region around the excretory pore, and the proximity of the large triangular cells, may indicate that these have some role in the overall function of the system. Certainly, the affinity of the triangular cells for vital dyes suggests an excretory role.

The hatching mechanism is an active process initiated by the miracidium. Rowan concluded that the principal factor was the release of a proteolytic hatching enzyme to dissolve the opercular seal. However, events may not be as simple as stated, and some of his experiments are open to criticism, particularly the section concerning "exosmosis of salts".

The viscous cushion is not, as Rowan supposed, a homogeneous gel. It has a definite fibrillar structure and possesses nuclei. Rowan did not mention these, though they are clearly seen in his plates. The origins and mode of formation of the viscous cushion are discussed in later parts. It is not present in schistosome eggs and Rees does not describe it in Parorchis. It is, however, present in Fasciolopsis (Barlow, 1925) and Cotylophoron (Bennet, 1936). I have personally observed it in Echinoparyphium and Echinostoma eggs and its presence may therefore be widespread. Rees figures the vitelline membrane of Parorchis as being thickened in the

region where one would expect to find the cushion. It is possible that its formation is in some way connected with the cells which break away from the embryo to form the vitelline membrane.

The results of the investigation of the nervous system were disappointing. This is possibly due to the fact that the constituent parts are too small to be resolved using the light microscope. Lynch (1933) observed several nerves emerging from the central mass in the miracidium of Heronimus but he was dealing with an organism up to 500 μ in length.

Apart from the eyespots no other sense organs have been substantiated. These must occur, however, as the miracidium is capable of perceiving such features as current direction, and of distinguishing between inert objects and intermediate hosts. The 'lateral bristles' could serve as rheoreceptors. These are likely to be found in an exposed situation and the miracidium possesses two such sites, the shoulders, and the apical papilla. In any other situation rheoreceptors would be obscured by cilia. The site of chemoreceptors has not been identified but one is forced to conclude that these lie in the anterior region possibly on the oral papilla. The actual penetration of the snail was shown by Dawes (1959, 1960 b, c) to be basically a chemical process of erosion of snail tissue. Observations have shown that the primitive gut is capable of secretion and this would imply that it was the main organ involved in penetration. The role of the so-called penetration glands is uncertain and in Fasciola they may have a reduced significance.

One is left with the impression that much remains to be discovered of the structure of the miracidium. Conventional techniques of histology, whilst still able to contribute, are not sufficient to solve the problem. The answer probably lies in the use of electron microscopy which Kümmel has used successfully to determine flame cell and eyespot ultrastructure. Electron microscopy was used later in this work to investigate shell and membrane structure, and the nature of the viscous cushion, but it was considered that a study of miracidial structure was outside the scope of this thesis.

The choice of five main fields of research was confirmed as a suitable basis for further work. It was felt that, since these covered all aspects of the biology of the egg and miracidium, they were more likely to lead to a balanced view than would preoccupation with a single feature.

PART III. THE STRUCTURE AND PERMEABILITY OF THE SHELL AND VITELLINE MEMBRANE.

Introduction.

The trematode egg shell is usually of a hard sclerotin-like nature, though some trematode families (Haploporidae, Zoogonidae, Cyclocoelidae, etc., Smyth and Clegg, 1959) are exceptions. These forms have thin or membranous shells and the egg develops in the uterus thus obviating the need for a protective outer covering.

The formation of the shell has attracted much attention, and a knowledge of the fluke reproductive organs is essential in understanding the mechanisms involved. The vitelline cells pass from the follicles along lateral collecting ducts to the central vitelline reservoir. The duct from the reservoir joins the oviduct to form a combined ovo-vitelline duct and a slight dilation of this denotes the ootype. This is surrounded by unicellular glands collectively called Mehlis' gland. Beyond the ootype, the ovovitelline duct joins the uterus and the junction is marked by a uterine valve.

Mehlis' gland was originally thought to secrete the shell and early workers gave it the name "shell gland". However, Leuckart(1886) concluded that the shell was formed from globules of material present in vitelline cells. This observation was supported by Henneguy (1906) and Goldschmidt (1909). Interest then centred around the composition of the globules. Vialli (1933) showed that they contained phenolic material, and

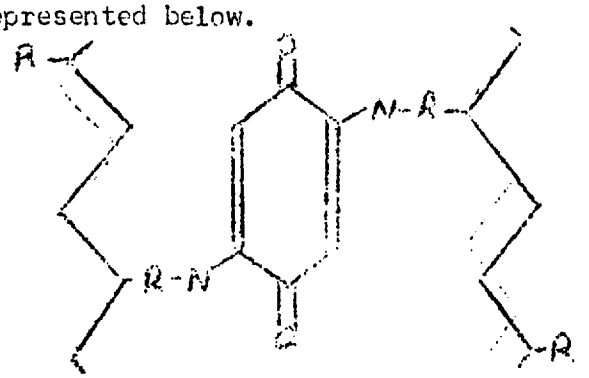
other components were demonstrated by Stephenson (1947) and Smyth (1951). The central role of Mehlis' gland was therefore rejected, though Yosufzai (1953) has revived the concept that this gland secretes the shell. He obtained a positive argentophily of the egg shell and Mehlis' gland but put forward no further substantiating evidence. Indeed, it has proved possible to stain the shell with many dyestuffs under appropriate conditions. The reasons for this probably lie, not in the chemical composition, but in the physical properties of such a system. The secondary functions proposed for Mehlis' gland are discussed later.

The concept of quinone tanning as a means of stabilising protein structures in biological material, was introduced by Pryor (1940 a, b) and extended to the egg shell of Fasciola by Stephenson (1947). Quinone tanning is widespread throughout the animal kingdom. Brown (1950) described it in molluscs, annelids, nematodes, and arthropods, and Nurse (1950) described it in the egg capsules of some Turbellaria. It is in fact found in the cuticle and egg cases of nearly all insects, and the mechanism of tanning has been most studied in these forms. Pryor's early work was substantiated by Trim (1941) and Fraenkel and Rudall (1947). Attention was then directed to the identification of the phenol precursor of the tanning agent. Pryor, Russel, and Todd (1946) identified the phenol of cockroach ootheca as protocatechuic acid. Hackman, Pryor and Todd (1948) examined a wider range of insects and described other phenols. Pryor (1955) also suggested that in some insects an alternative system might exist, utilising tryptophane derivatives. All investigations, however, seem to point unanimously to the

hardening of a cuticular protein by its combination with an orthoquinone which is produced by enzymic oxidation of a phenol.

Dennel (1958) has criticized the above system. He considers that the presence of protocatechuic acid in cockroach ootheca is not sufficient evidence for its being the tanning agent. There is also the point that protocatechuic acid will not tan protein in vitro. Dennel opens a second possibility, that para-tyrosine may give rise to the dihydroxy phenols, catechol, hydroquinol, pyrogallol etc., all of which will tan protein in vitro.

He extracted an unknown breakdown product from cuticle and identified it as an amino-phenol. The mechanism of tanning which he postulates is represented below.



The quinone is thought to be para-benzoquinone derived from hydroquinol, and not an orthoquinone derived from protocatechuic acid.

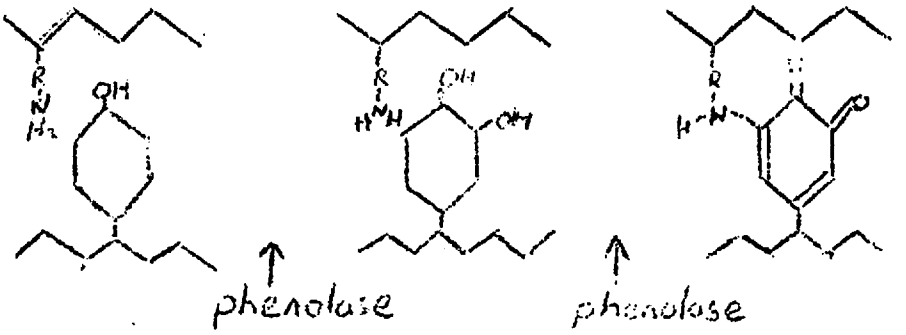
Attempts have been made in recent years to elucidate the exact tanning system which occurs in Fasciola. Most of the work has unfortunately been based on histochemical tests which are not sufficiently specific to completely characterize the reacting components. Vialli (1933),

Stephenson (1947) and Smyth (1954), all identified a phenolic component in the globules which could well be represented by the amino acids, tyrosine and phenylalanine. Attempts to extract free phenols such as those found in insects are said to have failed (Smyth and Clegg, 1959). A basic protein component was also demonstrated by Stephenson, Smyth, and Johri and Smyth (1956).

As the egg passes along the uterus it rapidly becomes negative to histochemical tests for phenols. This suggests an enzymic oxidation of phenolic material to quinones. Stephenson could not demonstrate the presence of phenolase but Smyth (1954) introduced a histochemical test for this enzyme and succeeded in locating it in the vitelline cell globules. The enzyme has been characterized by Mansour (1957, 1958). Thus, the three necessary components of a tanning system are present in the globules, and the question must be posed as to why premature tanning does not occur. Smyth and Clegg merely state that it is probably a blocking reaction of some kind.

The actual shell formation occurs in the proximal region of the uterus. In living specimens the uterus acts as a mould for the shell. The stimulus which causes vitelline cells to release globules was thought by Stephenson to be the presence of spermatozoa or possibly mechanical pressure caused by passage through the uterine valve. The actual oxidation of phenolic material to quinones may be mediated by the presence of a tissue haemoglobin which surrounds the proximal uterine region (Stephenson, 1947). The mechanism of tanning is still not clear. The idea of direct modificat-

ion of protein chains followed by cross linkage was first proposed by Brown (1950) and Blower (1950). The failure of Smyth and Clegg to extract free phenolic material led them to postulate that in Fasciola the mechanism consisted of the oxidation of protein tyrosine followed by the attachment of the modified molecule to a neighbouring protein chain through basic groups. Clegg (personal communication) states that he has also examined Schistosoma mansoni and reached a similar conclusion. His diagrammatic representation is given below.



The mechanism seems to be widespread among trematodes and has been reported by Gönnert (1955), Ma (1963) and others.

The function of Mehlis' gland is still in doubt. Various explanations have been put forward in addition to the one in which it is said to secrete the shell. Leuckart (1886) and Ujiie (1936) thought that the secretion was responsible for the release of globules from the vitelline cells. Dawes (1940) and Gönnert (1955) thought that it might form a limiting membrane inside which the shell solidified. Goldschmidt (1909) and Kouri and Nauss (1938) thought that its function was one of lubrication.

Rao (1959) studied the histochemistry of the gland and Clegg (1963)

characterized the secretion. He found a lipoprotein, sterols, triglycerides, and phospholipids; and in a personal communication (1965) states that he considers it to form the vitelline membrane. The glandular secretion mixes with the vitelline cells before the shell forms and then moves outwards to line the inner surface. The secretion also forms a membrane around the outer surface of the shell.

Clegg's findings on the origin of the vitelline membrane contradict the results of other workers. As mentioned in Part II, the general opinion is that cells break away from the developing embryo and come to lie beneath the shell, forming the vitelline membrane. Some variation in thickness of this membrane was noted in undeveloped and developed eggs (Plate 1a, 1b) and the situation is clearly in need of clarification.

The mechanism of shell and membrane formation represents only half of the problem, and the remainder, namely the structure and properties of the fully formed membranes, has not been studied. The subject therefore provided considerable scope for a detailed investigation using modern techniques. The work falls into three sections.

(a) An investigation of the structure of shell and membranes using the electron microscope.

(b) A detailed investigation of the chemical components of the shell and their characterization using spectrophotometry and chromatography.

(c) An investigation of the permeability of the shell and membranes using simple physical experiments, radio isotopes, and autoradiography.

Methods.

(a) Structure of the Shell.

i. Polarised light.

According to Stephenson, in optical sections the egg shell of Fasciola shows negative radial birefringence, and in surface view is isotropic. This arrangement is said to be "characteristic of concentric lamellae and was largely intrinsic in character". To confirm these findings, a polariser and analyser attachment were fitted to a conventional compound microscope. Shells were observed whole; in surface view; and as sections. The sections were prepared by freezing a suspension of eggs in water and cutting the block on a cold microtome at 1 - 2 μ.

ii. Electron microscopy.

Demands on electron microscope facilities were very great and their use was therefore limited. To obtain maximum information in the time available it was decided to concentrate on the fully developed egg. This of course left some undesirable deficiencies and more problems were created than solved. The techniques used were all standard practice in the laboratory at Ashurst Lodge and their application to the Fasciola egg required little or no modification. This resulted in a great saving of time.

Fixation: Buffered osmium tetroxide fixatives (Palade, 1952) were used throughout the investigation and gave good results. Fixation was carried out at a temperature of 0 - 4°C. for 30 minutes. Penetration of fixative

is a critical factor but the small size of the material reduced the difficulties normally encountered with osmium.

Dehydration: After fixation eggs were rapidly washed to remove osmium and the shells were punctured to allow dehydrating and embedding solutions to penetrate. The eggs were left overnight in 70% alcohol, then taken via 85% and 95% to absolute alcohol. Staining procedures were carried out during dehydration.

Impregnation and Embedding: Two embedding media were used in the investigation: Araldite and the very similar epoxy resin, Durcupan. The method of embedding using Araldite was introduced by Glauert and Glauert (1958) and modified by Luft (1961). It was further modified at Ashurst Lodge to give support for hard tissues and was therefore particularly suitable for Fasciola egg shells. The Araldite mixture consisted of Araldite M (10 ml.), Anhydride hardener (10 ml.), Accelerator (0.45 ml.) and Plasticiser (dibutyl phthalate) (0.45 ml.). Specimens were embedded as follows:

Two changes of 15 minutes each in Propylene oxide;

Three hours in propylene oxide/araldite mixture;

Overnight at room temperature in araldite mixture;

One hour in araldite at 35°C.;

Specimens transferred to embedding container;

Cured for 2 - 3 days at 60°C.

The procedure was modified for material stained in Phosphotungstic acid during dehydration. The eggs were transferred from absolute alcohol

to a solution of 0.01% Caustic soda in absolute alcohol (two changes of 15 minutes each). They were then washed in absolute alcohol and treated as above.

The Durcupan impregnation schedule was very similar but propylene oxide was omitted, a mixture of absolute alcohol/durcupan being used.

Block preparation: Two methods were used. Gelatine capsules were half filled with resin which partly solidified. The impregnated specimen plus resin were then pipetted to fill the capsule, a cap fitted, and air bubbles expelled. The resin was then cured.

A simpler and cleaner variation developed at Ashurst was preferred to the above method. Resin was poured into a shallow dish of aluminium foil and partially solidified. The resin containing the specimens was then poured on top, the specimens orientated and the block cured.

For sectioning, the capsules were trimmed and mounted on the microtome chuck. Small pieces of resin plus specimens were removed from the shallow dishes with a hacksaw, and mounted on pieces of dowel which fitted into the microtome chuck.

Staining: Staining techniques in electron microscopy involve the introduction of atoms of high electron density into the specimen. These are usually the heavier metals such as lead, bismuth, uranium, etc. Osmium itself has high electron density and combines with phospholipid membranes, and unsaturated bonds of lipids and proteins. The material was stained before impregnation with resin, in all cases. Phosphotungstic acid (1% in

absolute alcohol) was used for a period of 30 minutes. Alternatively, specimens were stained for 15 - 20 minutes in successive solutions of 1% uranyl acetate in 80%, 95% and absolute alcohol, according to the method of Finck (1960).

The method mentioned in Part II for staining sections for light microscopy, was frequently used as an adjunct to electron microscopy. The sections were removed from the microtome trough, to a microscope slide on a hot plate at 80°C., and stained in a solution of toluidine blue for two minutes. The slide was then washed in distilled water, differentiated in 50% alcohol, dried, and mounted in synthetic Depex.

Microtomy: Sections were cut on a Porter-Blum ultramicrotome using glass and diamond knives. Copper grids (200 mesh) coated with a formvar film were used to support the resin sections. Grids were examined in a Siemens Elmerskop II electron microscope operating at 80 K.V.

(b) Chemistry of the Shell.

i. Preparation of material.

Large quantities of clean fluke eggs were obtained from bile, washed several times and any small pieces of tissue, etc., removed. The shell material was then purified. 5 - 10,000 undeveloped eggs were transferred in about 5 ml. of water to a Potter-Elvejhem homogeniser, and the pestle section rotated slowly. The eggs were broken up and the shell reduced to small fragments. Two types of homogeniser were tried, an all glass model (Quickfit and Quartz Ltd.) and a hardened glass model with

teflon homogeniser piston (Camlabs Ltd.). The all-glass model wore out very quickly and contaminated the material with glass powder. The teflon model was far superior. Not only was contamination minimised, but the rolling action left shells fairly intact but with contents dispersed. To remove cellular debris, eggs were washed and slowly centrifuged in conical tubes several times, until a small pellet of purified material remained. This pellet was then used for experimental purposes.

ii. Spectrophotometry.

Hackman (1953a) discussed the ways of differentiating between melanin and sclerotin and states that faith can only be placed in spectrum differences. The liver fluke egg shell has a brown colouration and its spectrum was therefore investigated in an attempt to elucidate the structure. A Beckmann D.B. visible and U.V. spectrophotometer with an automatic scanning and recording device, was used. A Tungsten light source was used for wavelengths 800 - 400 mμ and a Hydrogen source for wavelengths 400 - 200 mμ. Purified shell material was further ground up in the teflon homogeniser to form a very fine suspension. The suspension was concentrated by centrifugation at 400 r.p.m. and resuspended in 2 ml. of water. The spectrum was then measured, and various other phenolic and related compounds such as melanin, p. amino phenol, etc., were observed for comparative purposes. Hypochlorite solution was also added to shell suspensions and its effect noted.

iii. Qualitative chromatography.

Purified shell material was hydrolysed in 6 N. Hydrochloric acid for 18 hours at 105°C. This treatment completely destroyed the resistant shells. The hydrolysate was concentrated in vacuo at 80°C., washed several times and the final residue dissolved in 100 µl of 10% Isopropanol. Some samples were desalted using a column of the ion exchange resin Zeo Carb 225 (Smith, 1960).

Two-way ascending chromatography, on Whatman No. 1 paper, was carried out on the hydrolysate. Papers were run overnight using Butanol/Acetic Acid/Water, and Phenol/Ammonia as solvents (Smith, 1960). Amino acids were visualised by spraying with 0.5% ninhydrin in acetone, followed by heating at 105°C. for ten minutes. Other special location reagents were used and details are given in the relevant text. Standard solutions of amino acids were used to construct a map of distributions and marker mixtures were included to assist in identification of spots. The spots were assessed visually to determine relative concentrations.

iv. Bleach extraction of shells.

Stephenson and Smyth have both recorded that a dilute solution of sodium hypochlorite completely dissolved the egg shell. It was considered that an investigation of the mechanism by which bleach acts and the products formed would indicate something of the structures involved.

Purified shell material was introduced into a stoppered tube together with 2 - 3 ml. of 10% 'Milton solution' (Sodium hypochlorite in

sodium chloride). The tube was shaken slowly for three to four hours to prevent sedimentation, and complete dissolution of the shell occurred. The extract was centrifuged and the clear supernatant was removed for purification. Desalting was not successful as a method of removing the Milton solution, and dialysis was therefore adopted. This was carried out overnight against tap water using a Visking tube, and was followed by further dialysis for one hour against distilled water. The dialysate was then concentrated by freeze-drying which resulted in a yellowish residue. This residue was taken up in distilled water and treated in three ways.

1) Spot tests to determine constituents, particularly amino acids (ninhydrin) and proteins and peptides (Bromophenol Blue, and Light Green).

2) Chromatography. Both one-way and two-way ascending chromatography were carried out, with ninhydrin as a location reagent.

3) Hydrolysis, followed by chromatography. The bleach extract proved extremely unreactive. It was therefore hydrolysed and a greatly increased ninhydrin-positive reaction resulted. The amino acid composition of the hydrolysate was therefore investigated by two-way chromatography.

iv. Quantitative estimation of amino acids.

The typical amino acid pattern of the shell was established by chromatography and quantitative estimation of the amino acids was attempted. The classical method consists of fractionating the hydrolysate using an ion-exchange column, collecting the elluate, and assessing the concentration colorimetrically. This requires expensive equipment which was not available.

Simpler but less accurate methods have, however, been developed and a densitometric technique was used here.

Aliquots of shell hydrolysate were chromatogrammed and amino acids visualised by dipping papers, in a standardised manner, through a solution of 0.2% ninhydrin. The spots were developed, cut out, and the intensity measured in an E.E.L. densitometer, using a blue filter. Standard concentrations of 0.01 M. Glycine were included and intensities of spots were plotted graphically. The area under each curve was estimated and the concentration was found by relating it to the standard Glycine. The intensity of the ninhydrin reaction varies slightly with different amino acids and the concentrations were adjusted using a series of factors given by Cramer (1955). The percentage composition of each amino acid was then calculated.

(c) Permeability of the Shell and Vitelline Membrane.

i. Permeability of the shell.

The only work to date, on the permeability of the liver fluke egg is a plasmolysis study by Rowan (1962), using a variety of organic compounds. His findings indicated that the shell was permeable to small molecules with a molecular weight of less than 150.

These results were reinvestigated using a homologous series of straight chain alcohols C_1 - C_4 at a 2.0 M. concentration. Plasmolysis studies have several inherent limitations and to assess the permeability of the shell a more direct method was used. Beament (1948) studied the

permeability of insect chorion by mounting eggs on glass tubes and observing rates of penetration. A similar procedure was used here. A microelectrode puller (Plate 6a) was used to draw out fine glass tubes to the required 15 - 20 μ external diameter. The shells of freshly hatched eggs were collected and placed in a desiccator for storage. With the aid of a Zeiss sliding micromanipulator the fine tube, previously smeared on the outer surface with a suitable adhesive, was inserted into the shell via the operculum (Plate 6b). The tube plus egg was mounted on a cork and stored for at least one week in an inverted specimen tube to allow the adhesive to set (Plate 6a). The permeability was tested using water, solutions of inorganic ions, and organic compounds. N.B. The manipulations involved in affixing the egg to the tube take several minutes, and slow drying glues must therefore be used. Substances such as Rubber solution, Durofix, Bostik, etc. dry too rapidly.

ii. Radio-tracer experiments.

Experiments on the permeability of the shell indicated that the 'vitelline membrane' was in fact the main barrier (i.e. the membrane present at the beginning of development and thought by Clegg to be secreted by Mehlis' gland, not the membrane which is said to be formed by cells breaking away from the embryo).

The permeability of this membrane was therefore assessed using radio-tracers. Inorganic phosphate labelled with ^{32}P was chosen as the most suitable isotope because of the simplicity in handling techniques. In

50 μ

Plate 6b. Tube inserted into egg.

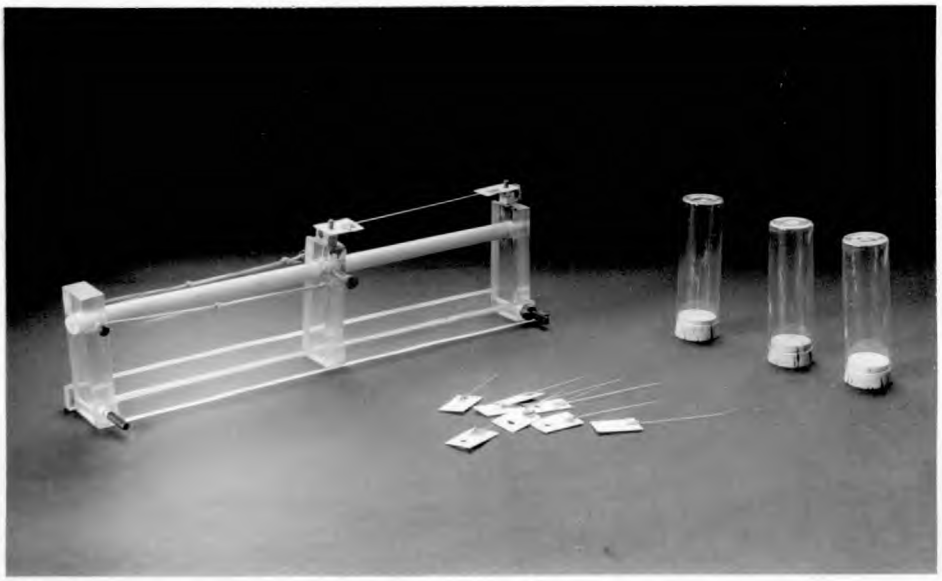


Plate 6a. Micro-electrode puller used in making fine tubes for insertion into egg.

addition the total phosphate content of the liver fluke egg could easily be measured and an idea of the rate and extent of uptake obtained.

A standard method of preparing samples of fluke eggs was used in all experimental work. Clean undeveloped eggs were suspended in distilled water, in a measuring cylinder. Several methods were used to obtain a homogenous suspension, and the most efficaceous was thorough mixing by inversion of the cylinder. 1 ml. samples were removed and counted in a McMaster chamber to determine the concentration per ml. Suitable aliquots of suspension could then be removed to flasks or tubes to give the required quantities of fluke eggs.

The colorimetric method of Allen (1940) was used to measure phosphate content, suitably scaled down, to detect as little as 0.1 µg of phosphorus. Total phosphate was determined by digesting tissue with perchloric acid to convert bound phosphate to the inorganic form. Bumping was prevented by addition of small chips of phosphate-free sintered glass. Free inorganic phosphate was determined on fresh tissue. Appropriate reagents were added to the cooled total phosphate digests and free phosphate samples to give a final volume of 0.5 ml. The blue colour which developed was read at 625 mµ in a Hilger "Spekker" using special 0.5 ml. cuvettes.

The greatest problem in all radio-tracer experiments is the removal of excess isotope after treatment. If this is not done efficiently then the amount of isotope taken up cannot be assessed. The problem is particularly acute in material such as fluke eggs which are susceptible to drying influences. After several attempts a method was devised for rapid

handling of samples with little or no damage.

The isotope was supplied as a solution of sodium phosphate (1 mg./ml.) and a specific activity of 1 m. curie/ml. The half life of ^{32}P is approximately fourteen days and the isotope was therefore used as soon as possible after receipt. Aliquots of the isotope were diluted in 0.02 M phosphate buffer (pH 7) to give an approximate count of 200 disintegrations/second/ml. (The necessary dilution factor could be calculated from the original activity, since 1 millecurie = 3.7×10^7 disintegrations/second).

Known quantities of eggs were pipetted into conical centrifuge tubes and concentrated. The supernatant was removed, and 1 ml. of an 0.02 M solution of diluted isotope was added. After varying time intervals eggs plus isotope were pipetted into special containers. These consisted of small copper tubes 1 cm long and 0.5 cm wide, with one end covered by 400-mesh phospher-bronze gauze (mesh size = about 45 μ). The gauze retained the eggs but the isotope passed through. The eggs were washed for 30 seconds in a continuous stream of 0.02 M phosphate buffer. Controls, without eggs, and with eggs and isotope mixed immediately before washing, were included with every experiment. To recover the isotope taken up, for scintillation counting, containers plus eggs were placed in small specimen tubes and two drops of absolute alcohol added. This destroyed cell membranes and allowed organic and inorganic constituents of the eggs to escape. To complete the process, the tubes were placed in the oven at 105°C. for fifteen minutes. 1 ml. of water was then added to each tube and

samples were left for 15 minutes to allow solution of ions to occur. 5 ml. of NE 220 scintillation fluid (Nuclear Enterprises Ltd.) was added to a vial, and background counts obtained. 0.5 ml. of the sample were then added to the scintillation fluid and five counts, each of 10 seconds duration, were carried out and averaged. Both concentration and activity of the Phosphate solution were known so that the amount of $^{32}PO_4$ which had been taken up could be calculated. Since the total and free phosphate content of the eggs were also known, it was possible to calculate the percentage exchange which had occurred.

The results of the tracer experiment indicated that the bulk of the isotope which had been taken up was in fact in the shell matrix. To test this hypothesis simple autoradiography experiments were performed. Eggs which had been in a solution of isotope for 3 hours were washed by the above procedure and small quantities were transferred to a microscope slide. They were then squashed to remove contents and dried quickly. The slides were placed in direct contact with Industrex D X-ray film and left for a suitable time duration, the position of the slides being marked with radio-active ink. The film was developed for about six minutes in Kodak D 19-b developer and fixed with Amfix. The position of squashed shells on the slides was recorded photographically and compared with the X-ray film to discover the location of radio-activity.

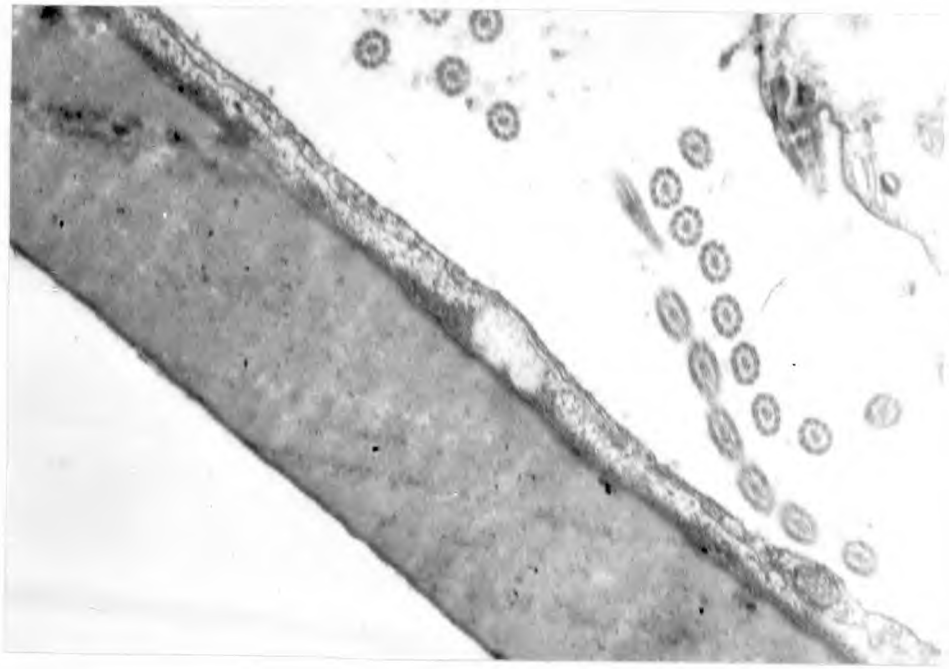
Results.

(a) Structure of the Shell.

Birefringence is the term used to describe the effect observed when a beam of polarised light passes through anisotropic material and the plane of polarisation is altered. In biological specimens this is brought about by the regular arrangement of 'particles' such as protein molecules.

In the intact egg shell of Fasciola there is, as Stephenson stated, a birefringence which he attributed to concentric lamellae. One would therefore expect the birefringence to be found in sections of the shell, but this does not occur. Neither is it present in surface views of shell fragments, and the apparent birefringence may therefore be an illusion. It could be caused by reflection from the convex surface of the shell which is highly polished.

The results obtained from electron microscopy were more encouraging. A section of the shell and membranes is illustrated in Plate 7. The egg was stained in Uranyl acetate prior to embedding and the magnification is × 30,000. The shell appears homogenous throughout, and there is no indication of the concentric lamellae proposed by Stephenson. (The diagonal marks visible are knife marks). The matrix of the shell in fact appears to be disorganised with random distribution of lighter patches of material. The outer 250 Å at both surfaces is considerably denser than the remainder of the shell, but the areas are not sufficiently defined to constitute separate layers. (A similar dense layer 200 Å thick was observed by Rogers (1956) in the chitinous egg shell of Ascaris). The areas in Fasciola may



0.5 μ

Plate 7. Electron micrograph of developed egg stained with uranyl acetate, showing shell, perivitelline material, and double vitelline membrane.

have an important effect on permeability.

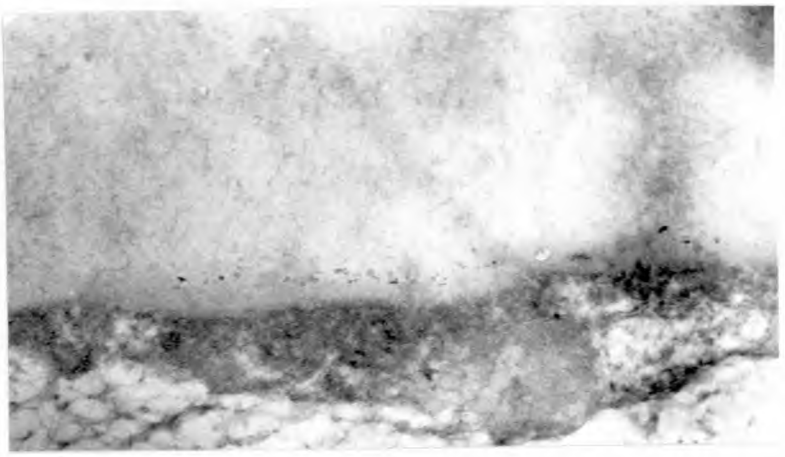
Lying directly beneath the shell is a layer of perivitelline material 1000 Å thick. It appears to be bounded on both surfaces by a very thin membrane, but has no apparent organization other than a greater density in its outer region.

Lying beneath the perivitelline material are two membranes which can probably be equated with the vitelline membrane of earlier workers.

These preliminary results were followed by an intensive investigation of the three systems to determine their exact nature and origins.

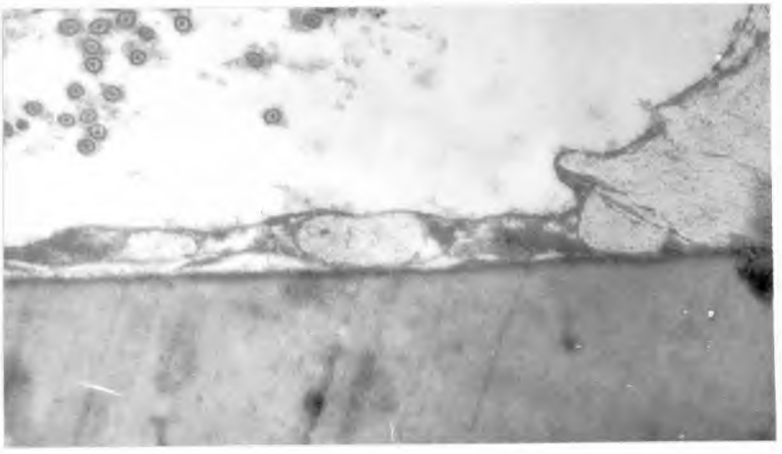
i. Shell.

The proposed mechanism of quinone tanning would seem to be a random process of cross linking, and as such would not give rise to an orderly structure at the electron microscope level. The shell stained with uranyl acetate, appears fairly homogenous but in eggs stained with phospho-tungstic acid and examined at a magnification of × 50,000 a faint structure can be discerned (Plate 8a). This takes the form of randomly orientated fibrils 200 Å × 25 Å, giving the whole a reticulated appearance. The background to the fibrils appears as a diffuse grey region but this effect could be produced by the thickness of the section or the presence of embedding medium. Minute dense granules could also be discerned in some parts, having a diameter of approximately 100 Å. They are possibly deposits of melanin or a similar aromatic polymer.



200 mμ

Plate 8a. Electron micrograph of shell stained with phosphotungstic acid to show reticulated structure.



0.5 μ

Plate 8b. Electron micrograph of developed egg showing viscous cushion lying within the double vitelline membrane.

ii. Pervitelline material.

This layer revealed by the electron microscope is probably the thin lining of the shell visible at the beginning of development, and hence identical with Clegg's vitelline membrane (see Part II). The name perivitelline material is proposed here for it, to avoid further confusion. Clegg describes the material as lipoprotein with other lipid constituents. If it is the main barrier to permeability, then it would be interesting to discover the mechanism by which it functions. The lack of apparent organisation may be the result of poor fixation, extraction of lipids by alcohol, or destruction during processing.

iii. Vitelline membrane.

The two membranes of which this is composed are separated by a distance of about 300 Å. This is filled with debris and inclusions of unknown function. The nature of these dual membranes can perhaps be explained if we consider their origins. The cells which break away from the embryo are said to form a complete lining to the egg immediately beneath the shell. The two membranes could then represent the surfaces of a flattened cell, and the inclusions, remnants of cytoplasm and cytoplasmic particles. Of great interest in this context is the origin of the viscous cushion. The investigation of its structure revealed that it was in fact formed between the two membranes (Plate 8b). This discovery is clearly compatible with the cellular nature of the vitelline membrane, and its origin from the developing embryo. These aspects are dealt with fully in the discussion.

iv. The operculum.

Repeated attempts, using the electron microscope, to find the operculum in intact eggs met with failure. The same problem occurred when sections were investigated using the light microscope. The opercular ring is thus not the prominent feature in sections, that it appears to be in whole eggs. Rupture of the operculum can be observed under the compound microscope. The disruption starts at one point and spreads round the opercular ring in the manner of a tear in a piece of material. The operculum is left with a jagged rim as a result of this process.

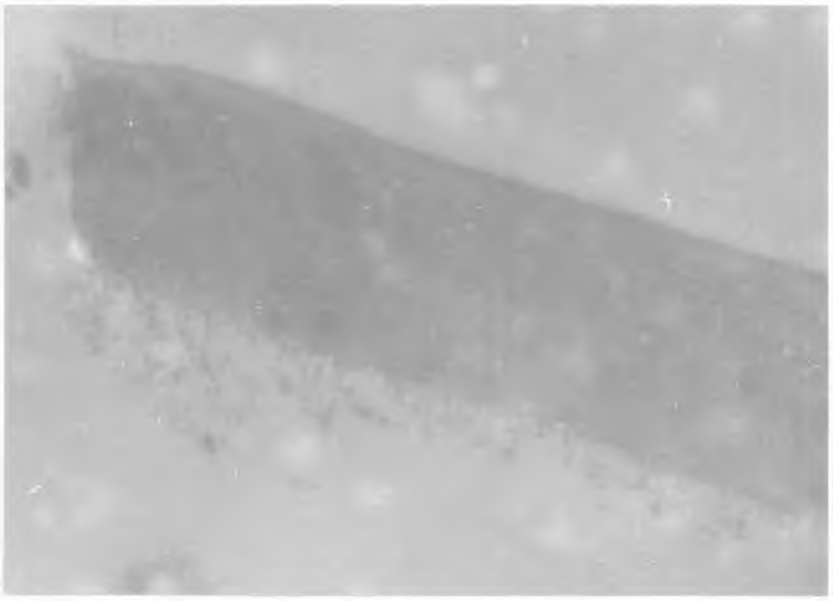
Electron micrographs of the hatched egg were not very informative (Plate 9). The fact that the operculum could not be easily located in sections of unhatched eggs suggests that it may be a simple discontinuity (either physical or chemical) on the inner surface.

One interesting point concerning the perivitelline material was evident in the electron micrographs of hatched eggs (Plate 9). The material had expanded and become extremely diffuse, probably by taking up water. This increase in volume suggested the presence of a mucoprotein constituent and this observation is supported by the fact that Clegg found that the secretion of Mehlis' gland contained 1% carbohydrate.

(b) Chemistry of the Shell.

i. Spectrophotometry.

As would be expected from the brown colouration of the shell, the absorption spectrum did not show any sharp bands. There was slight absorp-



0.5 μ

Plate 9. Electron micrograph of hatched egg showing ruptured opercular ring and expanded perivitelline material.

tion from 800 mμ decreasing to 700 mμ, and again general absorption from 500 mμ rising slowly to 200 mμ. The characteristics are those described by Hackman for sclerotin and we must therefore conclude that if melanin is present it is only in minor quantities.

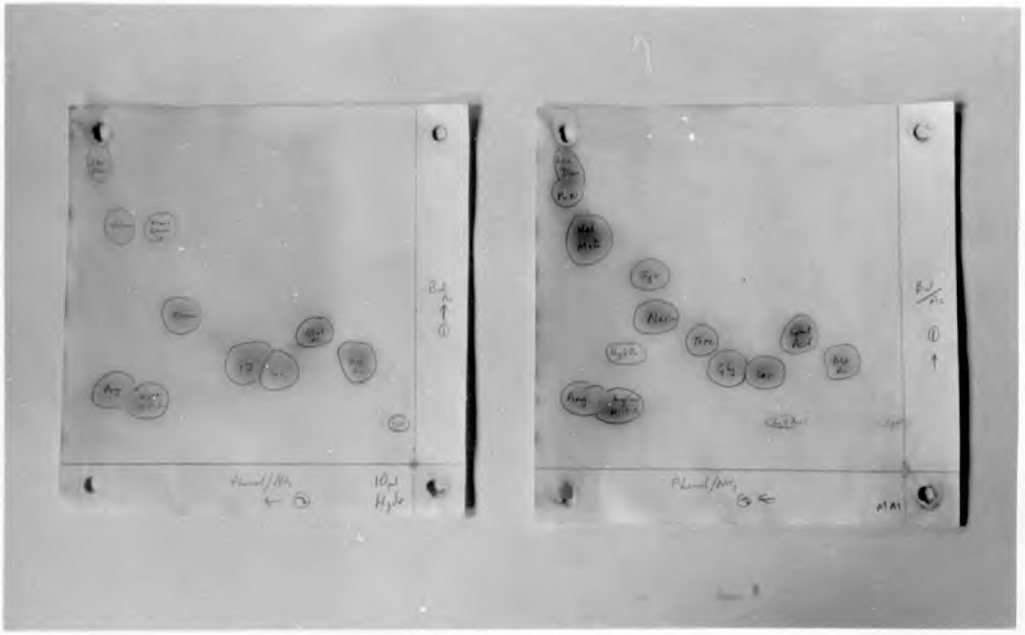
Addition of hypochlorite to the shell suspension resulted in a rapid disappearance of the absorptive regions. The spectrum of the bleach itself, however, obscured a considerable part of the shell curve.

ii. Chromatography.

Approximately ten amino acids were identified in the two-way chromatograms, an example of which is given in Plate 10. The procedure was repeated many times with identical results. The amino acid composition was assessed visually as follows:

Major components:	Glycine, Aspartic Acid, Glutamic Acid and Serine.
Minor components:	Alanine, Histidine, Arginine and Lysine.
Trace components:	Leucine or Isoleucine, Valine, Unidentified Spot, and possibly small quantities of Tyrosine and Cysteine.

Proline and Hydroxyproline were not detectable and the fact that only traces of tyrosine were found, is surprising. Attempts were made to characterise the unidentified spot by treating one-way and two-way chromatograms with a variety of location reagents. When sprayed with ninhydrin, it gave a bright green spot which rapidly faded to the characteristic pink-purple colour of an amino acid. With Ehrlich's reagent and Isatin results were negative but spraying with diazotised Sulphanilic acid gave an orange



Shell hydrolysate. Marker mixture.

Plate 10. Two-way chromatogram of shell hydrolysate visualised with ninhydrin to show constituent amino acids.

spot characteristic of Phenolic compounds (Plate 11). The R.F. of the compound in Butanol/Acetic Acid/Water, was determined as 0.57 compared with 0.61 for Phenyl Alanine, and 0.43 for tyrosine. Hydrolysates with added tyrosine yielded two discrete spots indicating that it was not being confused with that compound. Further characterisation would have required extensive amounts of material, and apparatus. On present evidence, however, the compound could possibly be an amino-phenol of some kind. It is of interest to note that a hydrolysate of insect egg shell produced an amino acid pattern very similar to that of the fluke shells. The 'aminophenol' spot was present, as well as glucosamine which was not detected in fluke shells.

iii. Bleach extraction of shells.

Spot Tests. Light Green and Bromophenol Blue tests for proteins (Smith, 1960) produced transient colouration of the test material. This colouration could be rapidly washed out in running water and it was concluded that the compounds involved were not of high molecular weight. Spraying with ninhydrin followed by heating at 105°C. for ten minutes resulted in a faint pink colouration of the test spots.

Chromatography. One-way chromatography indicated that several peptide fragments were present in the extract. Results were very variable and it was extremely difficult to remove all traces of bleach from the hydrolysate. The peptides did not react readily with any location reagent.

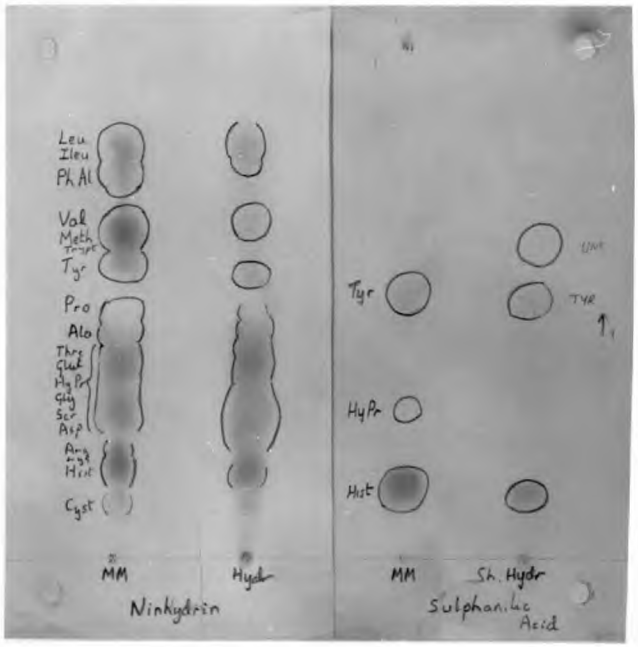


Plate 11. One-way chromatograms of shell hydrolysate visualised with ninhydrin for amino acids and diazo-sulphanilic acid for phenolic compounds.

Hydrolysis and Chromatography. Hydrolysis of the dialysed and concentrated bleach extract resulted in a great increase in reactivity with ninhydrin. Two-way chromatography of the hydrolysate showed that at least eight amino acids were present. These were identified as Aspartic acid, Glutamic acid, Glycine, Serine, Alanine, Histidine, Arginine and Lysine.

It was concluded, as a result of these investigations, that hypochlorite fragments the protein shell into several fractions. The peptides produced were of medium molecular weight (too large to pass through the pores of Visking tubing, and too small to be rendered insoluble by heat or acid treatment), having few reactive groups. The hypochlorite appears therefore, not only to reverse the tanning mechanism, as evidenced by loss of the absorption spectrum, but also to oxidise the protein chain at one or more points.

iv. Quantitative estimation of amino acids.

The densitometric technique used to estimate amino acid concentrations, proved the most reliable. With a glycine standard the method had an accuracy of approximately $\pm$ 5%.

Five concentrations of shell hydrolysate were used in the experiment. The spots were measured in the densitometer, and the area under each curve was taken as the total 'optical density' of that particular amino acid. The various optical densities were then plotted graphically, against concentration and lines were fitted through the points by eye (Fig. 6). An arbitrary concentration (6 µl) was selected and the figures were read off

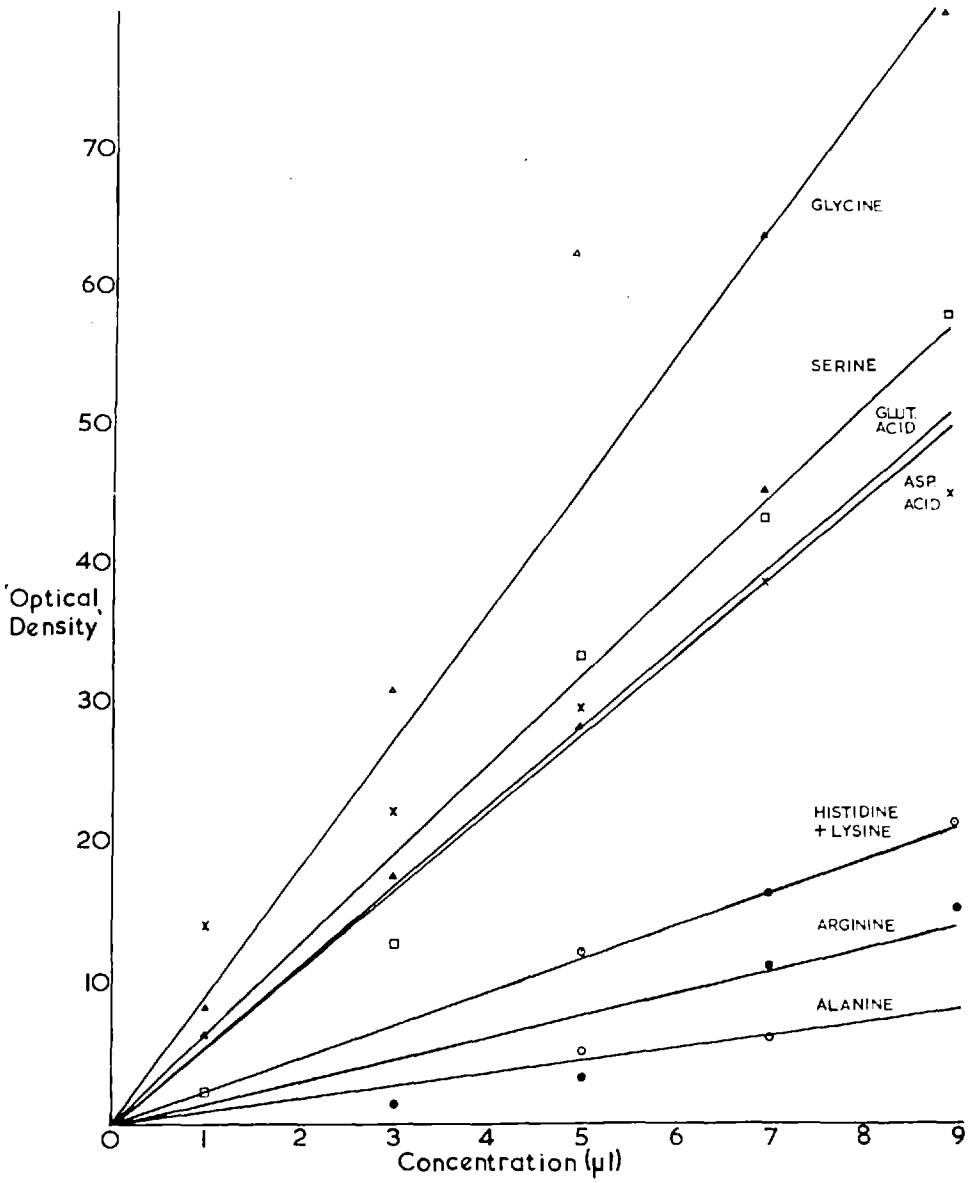


Fig.6 Optical Density of Shell Amino Acids

from the graph. These figures were corrected relative to Glycine, as described under Methods, and finally, the percentage composition of each amino acid was calculated. The results are given in Table I.

Table I.

Amino Acid	Optical Density	Corr. Factor	Corr. Reading	% Composition
Glycine	52.8	1.0	52.8	28%
Serine	37.0	0.95	39.0	20%
Glutamic Acid	34.1	0.93	36.6	19%
Aspartic Acid	33.3	0.93	35.8	18%
Lysine & Histidine	14.0	1.03	13.6	7%
Arginine	10.0	1.08	9.3	5%
Alanine	4.0	1.01	3.9	2%
Rest	-	-	-	circa 2%

(c) Permeability of the Shell and Vitelline Membrane.

i. Permeability of the shell.

Rowan (1962) demonstrated that compounds with a molecular weight of 150 or less could penetrate the egg shell of Fasciola fairly rapidly. Using 2 M solutions of alcohols $C_1 - C_4$ similar results were obtained. The times required for a solution to reach equilibrium (i.e. to replace by alcohol the water lost from egg thus reversing plasmolysis) were as follows: (mean of ten determinations) Methanol, 55 secs; Ethanol, 90 secs; Propanol,

203 secs; Butanol (saturated solution), 282 secs; i.e. a slower rate of penetration with increasing molecular weight and chain length. (The butanol molecule is approximately 8.5 × 3 Å).

The experiments utilising eggs fixed on tubes proved interesting. The tube plus egg was suspended laterally about 0.5 mm. above a microscope slide using plasticine as a support. A drop of test solution was pipetted on to the slide and the rate of entry into the egg observed under the microscope. Water penetrated in a matter of seconds over the whole surface of the egg. Unfortunately an air bubble was always trapped in the egg and the method could not therefore be used quantitatively. When eggs were removed from water, the rate of loss was more rapid, and all traces disappeared within one minute.

It was difficult to assess the rate of penetration of inorganic ions, but some indication of their penetration was gained in the following way. The egg shell was placed in the requisite salt solution for a period of time to allow diffusion to occur. It was then removed, washed rapidly, and immersed in a second salt solution which would precipitate the first (e.g. Sodium phosphate followed by Silver nitrate). It was not possible to observe precipitation inside the egg shell but in all cases examined, precipitation occurred within the shell matrix and resulted in an increased capacity.

Organic compounds of low molecular weight penetrated the shell quite easily but caution was exercised here as many of the solutions acted as solvents on the adhesives, weakening the seal, and entering the shell

via the operculum.

It must be concluded that the shell is permeable to water, inorganic ions, and small organic molecules. As plasmolysis experiments imply, it is not the main barrier to diffusion.

ii. Radio-tracer experiments.

According to Prosser (1962) "All freshwater eggs are hyperosmotic to their medium". Some means must therefore be provided to maintain the osmotic gradients prior to the development of excretory organs. It was not possible to identify this barrier with the shell, as is the case in insect eggs. The vitelline membrane, or the perivitelline material, or both, must therefore represent the barrier. Measurement of the uptake of labelled phosphate was considered to be a suitable method of testing the hypothesis since the shell was known to be freely permeable to this ion.

The total and free phosphorus content of the eggs were estimated colorimetrically. Each determination was duplicated and suitable standards and reagent blanks included. The total phosphorus content of 1000 undeveloped eggs varied from 1.0 - 2.02 µg. (mean of ten determinations = 1.57 µg). The free phosphate content varied from 0.15 - 0.20 µg (mean of four determinations = 0.18 µg/1000 eggs).

After several attempts, the isotope technique gave reproducible results. Uptake of phosphate was found to be very low in early experiments and a specific activity of approximately 200 disintegrations/sec./µl was finally chosen. For isotope experiments fresh eggs were used, completely

free from fluke or liver debris. The figures given below are the highest recorded in any experiment. Each determination was carried out in triplicate and four time exposures (0 hrs, 1 hr, 3 hrs and 24 hrs) were used. Each sample contained 5400 eggs. The isotope, when diluted, gave a count of 4554 disintegrations/sec./25 µl. i.e. a count of 182 disintegrations/sec./µl. Since the phosphate solution had an 0.02 M concentration, it contained 0.62 µg of P/µl and 1 µg of P was therefore equivalent to an activity of 293.3 counts/sec. The results, corrected for decay, are given in Table II.

Table II.

Time in Isotope	Activity (counts/sec.)	P taken up (µg)	Corrected Reading	µg P/1000 eggs
0 hours	13.4	0.05	-	0.018
1 hour	67.9	0.23	0.18 µg	0.066
3 hours	114.2	0.39	0.34 µg	0.126
24 hours	109.4	0.37	0.32 µg	0.120

Using these figures the percentage exchange (uptake) was calculated. In one hour there was an apparent uptake of P amounting to about 4.0% and in three hours, 8% of total P present. After twenty four hours, however, the figure was only 7.7%. These were the highest results recorded. It seemed likely therefore, that in fact little or no uptake had occurred and the activity was due to retention of isotope within the shells.

A simple experiment was performed to test this hypothesis. Eggs

were placed in a dilute solution of inactive sodium phosphate for three hours, removed and washed, and a drop of 1% silver nitrate then added. The shells of the eggs immediately began to darken as silver phosphate was precipitated. Control eggs left for three hours in distilled water, did not show this darkening of the shell, which must therefore be due to retention of phosphate within the shell matrix.

The results of autoradiography of whole and squashed eggs are illustrated in Plate 12. They demonstrate conclusively that the radioactivity is associated with the eggs and not due to residual isotopes remaining in solution. The squashed eggs caused extensive blackening of the X-ray film which is further evidence for the localisation of radioactivity in the shells. The extruded contents also caused some blackening, but to a markedly less degree. Sections of eggs examined by stripping film autoradiography would probably have clarified the matter. However, all attempts to cut frozen sections failed because eggs were invariably crushed by the freezing process. It is concluded therefore that the 'vitelline membrane' presents a barrier to diffusion of phosphate but that the matter is complicated by retention of ions within the shell matrix.

Discussion.

The purpose of the work in this section was to investigate the physical and chemical structure of the fully formed shell and 'vitelline membrane'. When the results obtained are compared with similar systems in other invertebrates, certain parallels can be drawn. Insect sclerotin

a

b

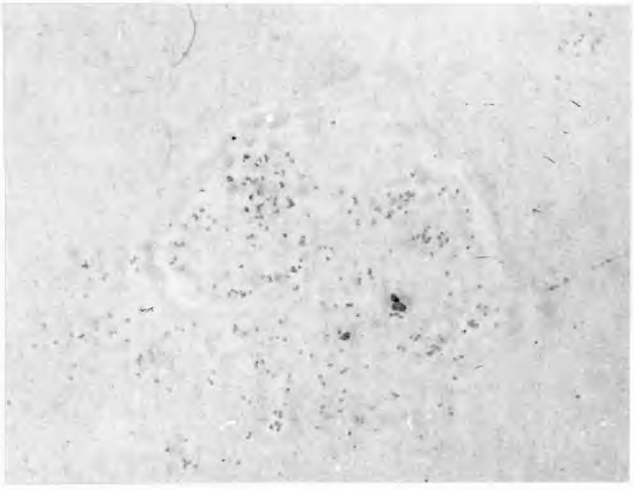


Plate 12. (a) Autoradiograph of fluke eggs immersed in $^{32}PO_4$ for 3 hours and then squashed on a slide.

(b) Photograph showing the distribution of fluke eggs.

and the fluke egg shell have both proved difficult to investigate. This can no doubt be attributed to the unusual properties of sclerotin which make it a successful piece of biochemical evolution.

Hackman (1953b) considered sclerotin to represent a whole family of closely related protein derivatives rather than a single compound. The presence of phenolic or quinone cross-linkages endows the protein matrix with great structural stability but the way in which it is constituted has not been successfully defined.

In Fasciola, experiments with polarised light indicated that the shell has a lamellar structure, as Stephenson stated. However, failure to observe birefringence in sections of egg shell, suggests that its appearance in the intact egg may be a surface-induced phenomenon.

The results of electron microscopy confirm this supposition. Division of the shell into layers was not observed, and the whole shell had a finely reticulated appearance, apart from the outer and inner 200 Å. The size of subunits (25 × 250 Å) is in the same order of magnitude as single protein molecules, or small numbers of molecules in the form of a microfibril. The subunits are orientated at random and it would be tempting to identify their junctions as sites of cross-linkage. The whole aspect requires further investigation, firstly with purified components extracted from Fasciola, and secondly with model systems of known configuration, before such conclusions can be drawn.

Many digene egg shells are colourless but supposedly consist of tanned protein. It was therefore thought that the brown colouration of

Fasciola shells could be due to melanin inclusions. On spectrophotometric evidence the composition of the shell is solely scleroprotein. Electron micrographs, however, indicate very small quantities of dark pigment which could be melanin or a similar polymer. The effect of its presence on overall colouration is, however, probably negligible.

The amino acid composition of the shell is of some interest. Smyth and Clegg thought that it might be collagen-like but the absence of proline and hydroxy-proline make this seem unlikely. The predominance of the four amino acids, Glycine, Aspartic Acid, Glutamic Acid, and Serine imply that prior to tarring the protein was of a simple fibrous type. There is little work with which a comparison can be made. Kreuzer (1953) identified ten amino acids in Ascaris egg shell, and Hackman (1953a) described a similar number in the insoluble fraction of insect cuticle. Unfortunately the work was not quantitative so their results cannot be interpreted.

It is not known whether the protein of the vitelline globules is a single homogeneous fraction. Clegg has extracted a phenol-rich substance from "vitelline glands" (presumably by homogenising parts of mature flukes). He attempts to identify its origins in the vitelline cell globules but details of his extraction procedure make it likely that his phenolic material is a mixture of peptide fragments containing tyrosine. It is not therefore possible to conclude that the vitelline globule protein was rich in tyrosine.

The virtual absence of tyrosine in the shell is of interest. If the mechanism of tanning proposed by Brown, Blower, and Smyth and Clegg, is the correct explanation, then its absence from shell hydrolysates would be

expected. It is possible that the unknown spot, tentatively identified as an 'amino phenol', represents the hydrolysis product of a modified tyrosine molecule.

The apparent violence of hypochlorite on sclerotin greatly diminishes its use as a tool in the study of tanning. Its effect is not only to reverse the tanning mechanism but also to oxidise the protein into miscellaneous and unreactive peptide fragments.

The structure of the opercular region remains a mystery and it is difficult to visualise how such a discontinuity could be formed. The only visible asymmetry of the undeveloped egg is the presence of the ovum near the opercular pole. This suggests a possible connection with the formation of the opercular discontinuity, but of course, any disruption of symmetry need only be at the molecular level to produce the desired effect. The difficulty in locating the intact operculum in sections and the way in which it 'tears' at hatching, imply that the discontinuity may extend only part way through the shell matrix.

Studies on the permeability of the shell confirm Rowan's supposition that small molecules pass through the matrix rapidly. Size of molecule seems to be the only determining factor as organic and inorganic, polar and non-polar molecules under a certain size all pass through easily. The size of the butanol molecule, which penetrates slowly, is approximately 9 x 3 Å and the 'pores' would therefore be a little larger than this. The central matrix has considerably larger spaces, about 200 Å in diameter, and we must therefore conclude that the limitations imposed on diffusion lie in the

inner and outer 200 Å of dense material. The pores of course would not be of the organised kind visible by light microscopy in the trout egg shell (Smith, 1957), rather, they would be in the form of spaces between adjacent protein chains.

The 'vitelline membrane' presents a more complex problem. The possibility of a dual origin became apparent during this investigation. It is necessary to postulate such an origin in order to take all previous work into account and at the same time satisfy the osmotic barrier requirements of the undeveloped egg. The terminology also requires clarification. In endolecithal eggs a vitelline membrane surrounds the undeveloped ovum, and as such the term should not be applied here to a membrane lining the shell. Its use is however maintained to describe the membranes said to be formed during development. The material lying between the shell and vitelline membranes is termed perivitelline material. If previous work on Fasciola and comparison with other fluke eggs can be relied upon then sufficient evidence is available on which to base an interpretation of the nature and function of the membranes. This reconstruction of events is speculative and represents a hypothesis on which to base further work. The scheme is illustrated in Fig. 7.

Clegg supposes that the secretion of Mehlis' gland mixes with vitelline cells, and after shell formation coalesces to form a lining to the shell. This lining is visible in the undeveloped egg (Plate 1a) and can probably be equated with the perivitelline material. Its function would be to present a barrier to diffusion in the early stages of the developing

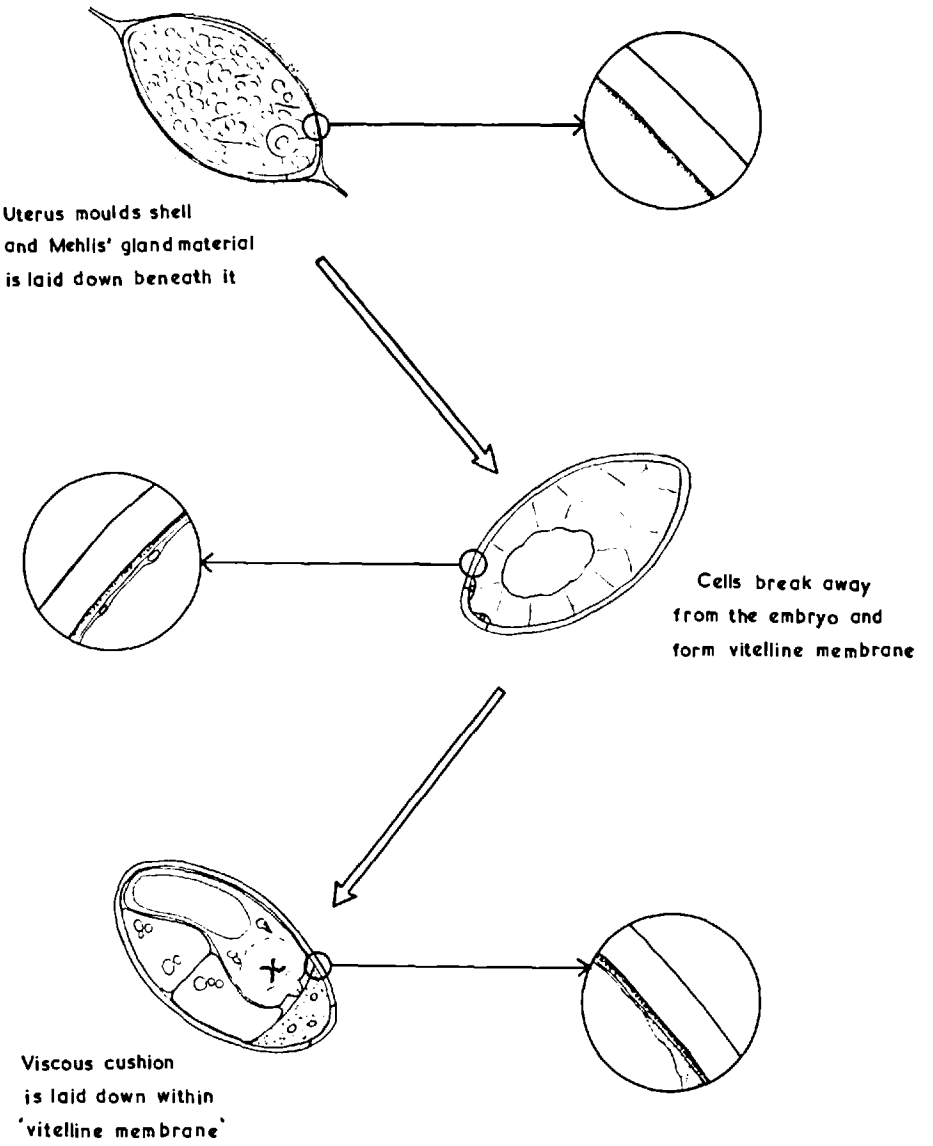


Fig.7 Formation of Shell and Membranes

egg. Later, cells break away from the embryo, pass to the opercular pole, and form a second lining to the shell. This is the vitelline membrane. Its double configuration is easily seen as a consequence of its cellular origin. Towards the end of development these cells lining the egg undergo an increase in metabolic activity and the viscous cushion is laid down within them. This explains the appearance of the viscous cushion between the two parts of the vitelline membrane (Plate 8b) and it is possible that its secretion is the main function of the extra-embryonic cells.

The chemical nature of the perivitelline material is unknown but Clegg described the secretion of Mehlis' gland as lipid, and lipoprotein with about 1% carbohydrate. The fact that it swells at hatching implies a hydrophilic nature. The only comparable work on egg membranes of invertebrates is that by Fairbairn and Passey (1955) on the vitelline membrane of Ascaris ova. This was found to consist of 75% lipid, mainly in the form of a C_{18} alcohol, ascaryl alcohol, and 25% protein. It represents the main permeability barrier in Ascaris eggs.

Radio-tracer experiments with $^{32}PO_4$ indicated that a similar barrier existed in the fluke egg. An uptake amounting to 8 - 9% of total phosphate in 24 hours was recorded. It is probable that the greater part of this was retained within the shell matrix indicating an uptake more apparent than real. Calculations based on shell volume show that an equal volume of active 0.02 M PO_4 would give a count in the same order of magnitude as that obtained in the experiments. Autoradiography of eggs also supports the presence of intense residual activity within the shell after isotope

experiments. It is of interest to note that Krogh and Ussing (1937) and Prescott and Zeuthen (1953) could not measure any penetration of deuterium oxide into trout eggs.

All freshwater eggs must be provided with some mechanism to maintain the osmotic gradient. Several theories have been put forward to explain the situation and the most widely held is that the vitelline membrane represents a differentially permeable barrier. The fish ovum has a similar organisation to the fluke egg. There is an outer, permeable, inelastic chorion, a space containing perivitelline material, and the ovum in the centre surrounded by the true vitelline membrane. Kao (1956a & b) has suggested that the colloid osmotic pressure of the perivitelline material compresses the ovum so that membrane pores are reduced in size. According to Prosser (1961) this mechanism seems to be present in marine and freshwater fish, and most other freshwater eggs investigated.

The perivitelline material in trout and frog eggs is mucoprotein in nature and hence, hydrophilic. In a recent paper Weiss (1963) discussed the effect of such hydrophilic substances present at the surfaces of cells. Two points mentioned are relevant to the above problem of permeability, namely the hydrodynamic properties of mucoprotein molecules and the kind of microenvironment which a thin layer of such hydrophilic, possibly polar molecules, would produce at cell surfaces.

Three alternative theories may be postulated to explain the situation in the fluke egg.

(a) The impermeability of the egg is controlled by a living membrane,

coupled to the metabolism,and capable of actively facilitating or preventing the passage of substances.

(b) There is an inert non-living barrier to diffusion, acting as a molecular sieve.

(c) That a hydrodynamic barrier exists, similar to that found in fish eggs.

In view of the supposed mode of origin of the perivitelline material, the first hypothesis seems unlikely at the beginning of development. The 'vitelline membrane', thought to appear in the middle of development, could however be of this type. The second possibility, of a molecular sieve, does not explain why the relatively large O_2 and CO_2 molecules are exchanged across the barrier whilst H_2O is not.

The third hypothesis therefore seems the most likely. One point in favour of this explanation is that when fluke eggs are placed in hypertonic solutions, water readily moves outwards across the barrier to equalise internal and external osmotic pressures. In eggs in distilled water, however, adjustment in the other direction is not observed under normal circumstances. This theory implies volume changes in the perivitelline material which should be discernible using the electron microscope. Further support for the theory is given by the fact that the perivitelline material increases in volume at hatching, and this suggests a hydrophilic constituent.

Certainly, the situation is complex and clarification must depend upon further work. It seems safe to state however that the egg of the liver fluke is similar to other freshwater types and falls loosely within that category which Needham terms "the cleidoic egg".

PART IV. THE CHEMISTRY OF DEVELOPMENT

Introduction.

The shell and membranes of the liver fluke egg represent a protective barrier within which the fertilised ovum develops. The process of embryonation requires extensive food reserves, the amount depending on the relative level of development before hatching. Investigations of the dynamics of the system constitutes the study of what Needham (1942) terms "Biochemistry and Morphogenesis". Such a study includes investigations of growth, utilisation of food reserves, cell differentiation, etc., and finally the relationship of all these to the fundamental biochemistry of the ovum/embryo.

This aspect of the biology of the liver fluke has attracted little attention. Ranzoli (1956) has described the development of the ovum and vitelline cells in the fluke, using histochemical techniques. He concluded that carbohydrate was the principal food reserve of the egg. Horstmann (1962) studied the oxygen consumption and glycogen utilisation of the developing egg and Bogomolova (1957) has described the histochemistry of the miracidium.

Among other parasitic metazoa, the eggs of nematodes have attracted attention. Fairbairn (1957) has reviewed the biochemistry of Ascaris ova, including respiration, and utilisation of food reserves. More recently the underlying enzyme systems have been studied by Costello et al. (1962, 1963), Oya et al. (1963a, b) and Costello (1964). These studies

have revealed some unusual features such as the role of the sugar trehalose in the metabolism, and the presence of quantities of 'ascarosides' within the egg contents and membranes.

The chemistry of the liver fluke egg was therefore investigated. The study was in the form of a preliminary survey as a true understanding of morphogenesis would require many years' work. The investigations fall into four sections.

(a) The chemical constitution of the vitelline cells, determined by histochemical means.

(b) The growth of the embryo and concomitant changes in dry weight of the egg.

(c) The respiration of the developing egg and of the miracidium.

(d) Changes in the chemical constituents of the egg during development.

Methods.

Quantitative measurements of biological material are affected by two inherent variations, firstly the accuracy of the technique used (a feature common to all experimental work), and secondly variations in the material itself. This latter feature is most prevalent in biological work. Thus light absorption measurements on Fasciola eggs, using a crude microspectrophotometer, indicated that variation between individual eggs may be ± 40%. To overcome this difficulty large samples of eggs (500 - 1000) were used in each experiment. The accuracy of the sample counting technique was in the order of 2 - 5%.

A third and more serious variation occurs in Fasciola eggs. This is, as Horstmann pointed out, the number of eggs which failed to develop. In the experiments reported here the number of embryonated eggs was always assessed and was found to vary between 65% and 85%. Comparison between experiments was therefore difficult and all results were corrected to 100% development for comparative purposes. The reasons for failure to develop are not clear but a certain number of the undeveloped eggs were later found to be infected with parasites (see Appendix I).

A fourth factor of importance in experimental work is the sensitivity of the technique employed. This is relatively unimportant when large quantities of uniform material are available, but becomes increasingly important when the material is limited, and microchemical techniques are adopted.

Whilst the effects of all these variations are not cumulative, it is doubtful whether an overall accuracy of more than ± 5% was attained in any particular experiment. In some cases this variation was as high as ± 10%. Interesting metabolic changes might therefore be missed.

(a) Histochemical Techniques.

In order to make preparations of vitelline cells suitable for histochemical studies, undeveloped eggs were fixed in Baker's Formaldehyde for several days. A small number of eggs were transferred to an albumen-smeared slide, and concentrated. They were then gently squashed under a coverslip and the vitelline cells freed. A drop of formalin fixative run under the

coverslip and left for five minutes, caused the cells to adhere to the slide. Squashes of eggs at different stages of development were also prepared.

The presence of nucleic acids was assessed using the methyl green-pyronin technique. The original method by Brachet (1942) and the modification by Kurnick (1955) were both employed. The distribution of protein was studied using the Mercuric chloride-Bromophenol Blue method introduced by Mazia et al. (1953).

Several reactions were utilised to locate carbohydrates, principally the Periodic Acid-Schiff (PAS) technique of McManus (1946) for polysaccharides (other than acid mucopolysaccharides). Glycogen was located using Best's Carmine method (Best, 1906). The occurrence of acid mucopolysaccharides was investigated using the Alcian Blue and Toluidine Blue techniques (Pearse, 1960). This latter test can be used on fresh and fixed material and is also useful in locating nucleic acids. In addition the stain has some affinity for mitochondria (see Part II).

The distribution of lipids during development was studied by staining formalin fixed material with a saturated solution of Sudan Black B. Temporary preparations were made and results recorded immediately. In order to characterise the lipids present, the Nile Blue method for neutral and acidic lipids (Cain, 1947) and the Nile Blue method for Phospholipids (Menschik, 1953) were used. The results were assessed from temporary preparations.

(b) Growth and Weight Determinations.

Classical studies on vertebrate chemical embryology have been facilitated by the fact that growth is relatively easy to assess. The embryo can be separated from the egg contents and analysed alone. In fluke eggs this direct course is not possible, which necessitates the use of more devious methods to measure growth. The simplest technique involves measurement of growing embryos and relation of the dimensions to actual volume by the use of some simple physical formula. In this case, length and breadth of a random sample of embryos were measured at daily intervals. The embryo, at least during the first three-quarters of development, has an approximately ovoid shape. It can be most nearly described as a prolate spheroid, and the data obtained were therefore used to calculate the approximate volume by applying the formula $V = \frac{4}{3} \pi ab^2$, where a and b are the major and minor semi-axes respectively.

Many elegant techniques have been devised for measuring the weight of small amounts of material. One of the principal methods is the use of a glass fibre under torsion. The balance illustrated in Plate 13 and based on that of Lowry (1944) was used to determine fresh and dry weights of undeveloped eggs.

Several balances were constructed covering the range 10 µg to 5 mg. and calibrated using small platinum weights. Each balance was covered by a glass aquarium tank during weighing. The weighing boats consisted of $\frac{1}{2}$ cm. circles of 400 mesh phospher bronze gauze with a fine glass loop for attachment to the balance beam. Using a pipette, samples of eggs in

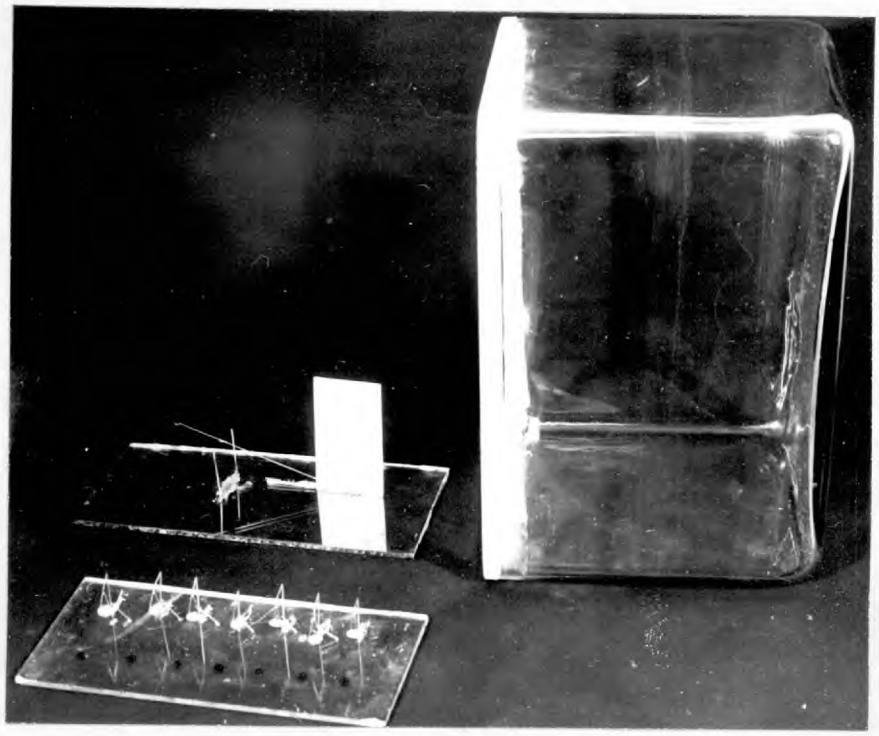


Plate 13. Microbalance and rack holding weighing grids.

suspension were transferred to weighed grids, which rested on a pad of absorbent paper. The excess water was removed by gently placing the grid on clean dry blotting paper, and the grid plus eggs was then rapidly weighed. Dry weights were determined by placing the grids on the rack shown in Plate 13, and drying overnight at 105°C. The grids were then reweighed and dry and fresh weights calculated.

The balances constructed in the laboratory had a sensitivity of about 10 µg and daily changes in dry weight/1000 eggs during development were less than this figure. A Cahn microbalance was therefore used for these determinations and glass loops were removed from weighing grids. A 1 mg. scale was selected and, since each grid had a weight of about 5 mg., this entailed the use of a 4.5 mg. counterbalance. The sensitivity of the Cahn microbalance is 0.1 µg., and of the technique used, ± 1 µg. Contamination of weighing grids with dust, debris, etc., was a serious problem and they were therefore placed in 1 cm. diameter wells in special perspex trays, the whole tray being enclosed in a petri dish.

Eggs were transferred to the grids using the technique mentioned above and after drying, the petri dishes containing trays plus grids were cooled in a desiccator to prevent condensation. All grids were handled with fine forceps.

In the experiments performed, samples of 1000 eggs were incubated in 50 ml. conical flasks at 25°C., and at daily intervals several flasks were removed to the refrigerator at 4°C. to arrest development. The washing, drying, and weighing of eggs were then carried out under uniform conditions

at the end of the experiment.

The dry weight of shells was determined by collecting samples of hatched eggs, washing and treating as above. No correction factor was applied to allow for the absence of the operculum.

The dry weight of miracidia proved more difficult to determine as they swam through the meshes of the grid during manipulation. This difficulty was overcome by killing the miracidia with heat or a drop of 5% formalin and then transferring to the grids. This of course introduces considerable error and results can only be approximate. (A possible means of overcoming the difficulty might be to use millepore filters followed by drying in a desiccator).

(c) Respiration.

The Warburg technique used by Horstmann for measuring oxygen consumption of fluke eggs requires considerable amounts of material. A variety of more sensitive microrespiration techniques have been devised and the subject was reviewed by Tobias (1943). These techniques include the well-known Cartesian diver method of Linderstrom-Lang (1937) and the less familiar capillary respiration techniques of Tobias and Gerard (1941) and Cunningham and Kirk (1942). The two methods have approximately equal sensitivities and each possesses certain advantages. The capillary technique used here has the simplest apparatus which facilitates manipulation of material The apparatus used is a design by Dr. F. Call of Imperial College, based on the method of Tobias and Gerard.

The apparatus was mounted in a constant temperature water bath surrounded by suitable lagging, and the temperature was controlled by the use of Techne Tempunit having an accuracy of $\pm$ 0.05°C. The microrespirometer is illustrated in Plate 14. The biological material was placed in 100 µl flasks with ground glass necks. A section of coarse capillary (1.0 mm bore x 1 cm long) fits into the neck of the vessel and is connected to a length of high quality fine bore capillary (0.10 mm bore). The assembled unit is inserted into the frame, which is in turn inserted into the large glass chamber. The chamber is mounted vertically in the C.T. bath and the frame can be rotated to allow inspection and measurement of capillary tubes.

The procedure used in setting up an experiment was as follows:

i. Respiration vessels and capillaries were washed in ether, acetone, water, acid cleaning mixture (overnight), water, acetone and ether to ensure that they were scrupulously clean.

ii. Washed eggs were transferred to respiration vessels and excess water removed to leave samples suspended in approximately 10 - 15 µl of water. The necks of vessels were then dried with tissue.

iii. The ground section of the capillary was lightly smeared with grease (Edwards Silicone, High Vacuum) and a piece of Whatman No. 50 filter paper (1 mm x 10 mm) was inserted into the coarse bore. In those tubes in which oxygen uptake was to be estimated, 1 µl of 20% potassium hydroxide solution was applied to the paper. The capillary was then inserted into the respiration vessel and the joint sealed.

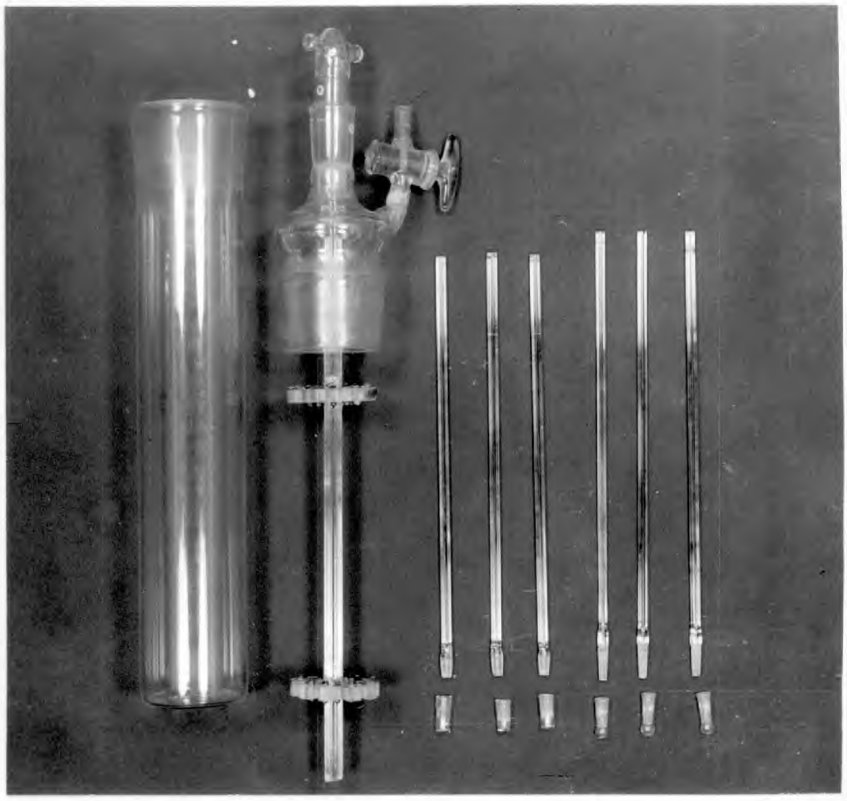


Plate 14. Microrespirometer apparatus.

iv. When all ten tubes had been prepared in this way, the respiration indicator fluid (2.7. dimethyl octane) was introduced into the capillary. This was effected by heating the vessel between the fingers for twenty seconds and then applying a drop of the fluid to the open end of the fine capillary. As the vessel cooled the droplet was drawn into the capillary to form a column 2 - 5 mm long.

v. The frame was then placed into the large vessel and the joint sealed with silicone stopcock grease. It was equilibrated for one hour with the tap open to allow expanding air to escape. The tap was then closed and the first reading (Time 0) was taken after five minutes. The movement of respiration fluid/measured from a reference mark on each tube, using a Vernier microscope reading to 0.02 mm.

The respiration of developing eggs, and miracidia, was investigated using this elegant technique.

Developing eggs: Fresh eggs obtained from mature flukes were incubated at 25°C. and the rate of respiration of samples was measured daily during embryonation (11 days). All the experiments were carried out at 25°C. The ten tubes available were divided into two equal groups. The first, used to measure total oxygen consumption, consisted of three tubes with samples and two tubes with water only (to act as controls, i.e. thermobarometers). All five had filter paper inserted into the coarse capillary and soaked in potassium hydroxide.

The second group were used to measure changes in total gas volume (O_2 taken up minus CO_2 given off). Three tubes contained samples and two

water. Potassium hydroxide was omitted.

After an initial equilibration period of 65 minutes readings were taken at 15 minute intervals for three hours. The apparatus was then dismantled and the respiration vessels removed. The eggs were transferred to a McMaster counting chamber using a fine pipette and the total present (usually around 1000 per tube) was counted. The percentage development was also determined.

The results obtained were in terms of millimetres moved by the meniscus of the indicator fluid. They were corrected for variations in the thermobarometer tubes and then converted into microlitres by applying the formula, 1 mm moved = 0.03 µl. This factor was determined by introducing a bead of mercury into the fine capillary, measuring its length with a Vernier microscope, expelling the mercury, and weighing it accurately. From the weight and density the volume of the measured length could be calculated, and hence, the volume of a 1 mm section of tubing. The RQ and QO_2 were then calculated for each day of development.

Miracidia: No suitable technique was devised for concentrating miracidia to enable their transfer to respiration vessels. The rate of respiration was therefore measured in the presence of some unhatched eggs. Fully developed eggs were transferred to the vessels and the apparatus assembled. The miracidia quickly hatched and, after thirty minutes equilibration, the experiment was started.

Several features were investigated, including the determination of oxygen uptake, QO_2 and RQ. The change in rate of oxygen consumption with

time and the effect of alternate light and dark periods on oxygen uptake, were also determined (Horstmann noted that periods of darkness depressed oxygen consumption). At the end of each experiment the miracidia were killed with formalin, removed to a chamber and counted.

Correction factor: When potassium hydroxide is present in the tubes, the meniscus measures the true rate of oxygen consumption. If, however, potassium hydroxide is omitted and carbon dioxide is evolved, a correction factor is necessary to account for the carbon dioxide dissolved in the medium present in the chamber. The differential microrespirometer used here works on the principle of constant pressure and changing volume, and the normal Warburg correction could not be used. A suitable factor was therefore devised.

$$\text{The factor } K = 1 + \frac{\alpha vf}{V}$$

where α is the absorption coefficient (0.76 at 25°C., for CO_2)

vf is the volume of fluid present (µl).

V is the volume of the gas space = volume of chamber - vf.

Here, putting in typical values, $K = 1 + \frac{0.76 \times 15}{115}$

$$= 1.1$$

The calculated values of carbon dioxide evolution were therefore multiplied by this factor to give the true reading.

(d) <u>The Chemistry of Development</u>.

The aims of this section of the work were firstly to determine quantitatively the food reserves present in the undeveloped egg and secondly

to discover the way in which these were utilised in the formation of the embryo. Nitrogen (Protein), Carbohydrate, Lipid and Nucleic Acid content of undeveloped and developing eggs were assessed.

i. Protein metabolism.

Histochemical techniques confirmed that abundant quantities of protein were present in the undeveloped egg. The shell was also known to consist of protein and its weight had been determined. Total Nitrogen content was selected as a measure of protein present, and methods of determining this were investigated.

The microdiffusion method of Conway (1947) has a sensitivity of 5 - 10 µg. The amount of Nitrogen present/1000 eggs was somewhat less than this and a colorimetric method based on Nessler's Reagent was finally adopted. A great many difficulties had to be overcome before a reproducible technique was developed. Glycine was used as a standard source of Nitrogen during the preliminary experiments, and the effect of digestion time, catalyst, neutralisation, amount and method of addition of Nessler's reagent, etc., were all investigated. The sensitivity of the method developed was about 1 µg with an accuracy of ± 8%.

The method was based on the procedures of Levy (1936), King (1951), and Ballentine (1957). The apparatus designed by King and manufactured by Gallenkamp Ltd., was used. The sample was concentrated in the bottom of special digestion tubes and 0.1 ml of concentrated Sulphuric acid (M.A.R., B.D.H. Ltd.) was added, also, a small piece of selenium catalyst tablet and

a piece of sintered glass to prevent bumping. The tubes were digested for 1.5 hours (optimum), cooled rapidly, and 5.0 ml of 0.64 N Caustic Soda solution were added to each, giving the digest a pH of about 6. Nessler's Reagent (0.5 ml) was then added in a standard manner, based on the method of Levy. A stream of filtered air was directed into the neutralised digest using a glass capillary. Thorough mixing ensued and the Nessler's Reagent was then blown into the digest mixture. Air was bubbled through for a further fifteen seconds and the capillary was then withdrawn. The tubes were left for ten minutes and the optical density of the solutions was determined at 425 mμ using a Hilger Spekker.

The Nitrogen content of undeveloped eggs, embryonating eggs, miracidia, and empty shells were all determined. Water blanks and Glycine standards were included in each experiment which was usually performed with five replicates.

The soluble protein content of undeveloped and embryonating eggs was also determined by the method of Lowry et al. (1951). The soluble protein was extracted by homogenising samples of 5000 eggs, transferring the homogenate and washings to a graduated tube, and making up to a volume of 5 ml. The tube was then centrifuged at 4000 r.p.m. for 5 - 10 minutes and 1 ml aliquots of supernatant were assayed for protein content. A standard made up from freeze-dried serum (Burroughs-Wellcome) and having a protein content of 100 μg/ml was used in the colorimetric procedure. The optical density of the solution was determined at 750 mμ using the Hilger 'Spekker'.

A freeze-dried protein extract of undeveloped eggs was electro-

phoresed to test its homogeneity. The Shandon horizontal apparatus was used in conjunction with cellulose acetate paper (Oxoid Ltd.). Procedures were standardised as follows: 2.5 x 12 cm cellulose acetate strips were soaked in buffer solution and placed across the bridge of the apparatus, tension being applied by means of stripholders. The current was switched on and papers were left for one hour to equilibrate. The sample of protein in a minimum of water, was applied as a streak to the centre of the paper using a capillary pipette. Electrophoretic separation was carried out at room temperature and runs were usually of one hour's duration. In this particular experiment an 0.05 M Barbitone buffer (pH 8.6) was used. A potential of 10 volts/cm strip length gave a current of 1 mA/cm strip width. After each run, the strips were dried at 105°C. for five minutes and the protein was visualised using a Mercuric chloride - Bromophenol Blue stain (Smith, 1960).

Attempts were also made to detect nitrogenous excretory products released from the egg during development, and present in the egg at the end of development.

ii. Carbohydrate metabolism.

Horstmann (1962) noted the presence of glycogen in the egg of the liver fluke and measured its utilisation during development. His work was repeated here using a different method to determine total carbohydrate content. Many such methods have been described and their specificity varies widely. The Anthrone techniques of Kahan (1953) and Scott and Melvin (1953),

and the Arsenomolybdate method of Nelson (1944) modified by Somogyi (1952) were investigated. The first of these was selected because of its apparent specificity for carbohydrates, and its greater sensitivity. The carbohydrate was extracted from a sample of eggs by homogenisation in 5% Trichloro-acetic acid, followed by centrifugation to remove the protein precipitate. Aliquots (1 ml) of this supernatant were transferred to one-inch-diameter test tubes and assayed for carbohydrate. The optical density of the green solutions was determined at 625 mμ in the 'Spekker'. Glucose standards (100 μg/ml) were included in all experiments. The range of the method was from 5 - 200 μg of carbohydrate, with an accuracy of ± 3%.

iii. Lipid metabolism.

Results of the histochemical investigation indicated that the lipid component was predominantly neutral lipid. Neither acidic nor phospholipids were detected. A variety of micromethods for the determination of lipids were investigated, including the microtitration procedure of Schmidt-Nielsen (1942); the Sudan Black densitometric method of Swahn (1952); and the colorimetric method of Rapport and Alonzo (1955). This third technique was adopted, suitably scaled down to give a sensitivity of 5 μg of lipid and a final volume of 1.0 ml.

The lipid material was extracted from eggs by homogenising in an alcohol-ether (3 : 1) mixture. The homogenate was transferred to a water bath at 60°C. and left for one hour. It was then centrifuged and the supernatant removed. The residue was re-extracted with alcohol-ether and finally

twice with ethyl ether. The four supernatants were combined and evaporated to dryness. The lipid was resuspended in 5 ml of absolute alcohol and the lipid content of 1 ml aliquots was determined. A standard of Glycerol tripalmitate (100 µg/ml) was used. The optical density of the purple solution was determined after thirty minutes, at 525 mµ in the 'Spekker'.

iv. Nucleic acids.

Histochemical tests indicated a great increase in the amount of nucleic acid present, as the embryo developed. Attempts were therefore made to measure the nucleic acid content of the undeveloped and fully developed eggs. Purification of the nucleic acid was carried out as follows. 5000 eggs were homogenised in 10 ml of ice cold 5% perchloric acid, centrifuged, and re-extracted. The supernatants were discarded and the residue treated with 3 ml of 5% perchloric acid, for 15 minutes at 80°C. (with constant stirring). The solution was centrifuged, the supernatant removed, and the residue re-extracted with a further 2 ml of perchloric acid, as above. The two supernatants were combined and made up to 5 ml in a volumetric flask. This constituted the nucleic acid extract.

The absorption spectrum of aliquots of the extract was determined using the Beckman D.B. spectrophotometer. Varying concentrations of a standard of mixed nucleic acid bases were also determined and a calibration curve constructed. The nucleic acid content of the eggs was then calculated.

Results.

(a) Histochemical Techniques.

Sections through the vitellaria of mature flukes, stained by the Periodic acid - Schiff (PAS), and Bromophenol Blue techniques revealed the situation as described by Ranzoli (1956), Smyth and Clegg (1959) etc. The principal components of the vitelline cells were an outer layer of proteinaceous globules, surrounding a clear cytoplasmic area containing much carbohydrate (Fig. 8a).

The vitelline cells visible in the undeveloped egg had a more orderly structure with complex aggregations of vacuoles, granules, etc. (Fig. 8b). This organisation was readily destroyed by fixatives which may account for the lack of structure shown by cells in situ in the vitellaria. The distribution of particular constituents is described below.

i. Nucleic acids.

The vitelline cells contained prominent DNA-positive nuclei but little detectable RNA in the cytoplasm. On the other hand the ovum/embryo contained large amounts of RNA uniformly distributed throughout the cytoplasm. This was particularly marked during the period of cell division but decreased during later cell differentiation. (The cytoplasm of the germinal cells was an exception to this). The reduction in RNA concentration is presumably paralleled by increased protein synthesis.

ii. Protein.

The vitelline cells contained dense aggregations of protein

Fig. 8. Chemical Constitution of Vitelline Cells.

a. Follicle of vitellaria stained by PAS procedure showing carbohydrate and protein shell globules.

b. Unstained vitelline cells.

c. Vitelline cell stained by the Mercury-Bromophenol Blue procedure for protein.

d. Vitelline cell stained by PAS method for carbohydrate.

e. Vitelline cell stained by Best's Carmine procedure for glycogen.

f. Unfixed vitelline cell stained with Sudan Black B, showing lipid droplets.

g. Vitelline cell stained with Toluidine Blue showing granules (mitochondria?).

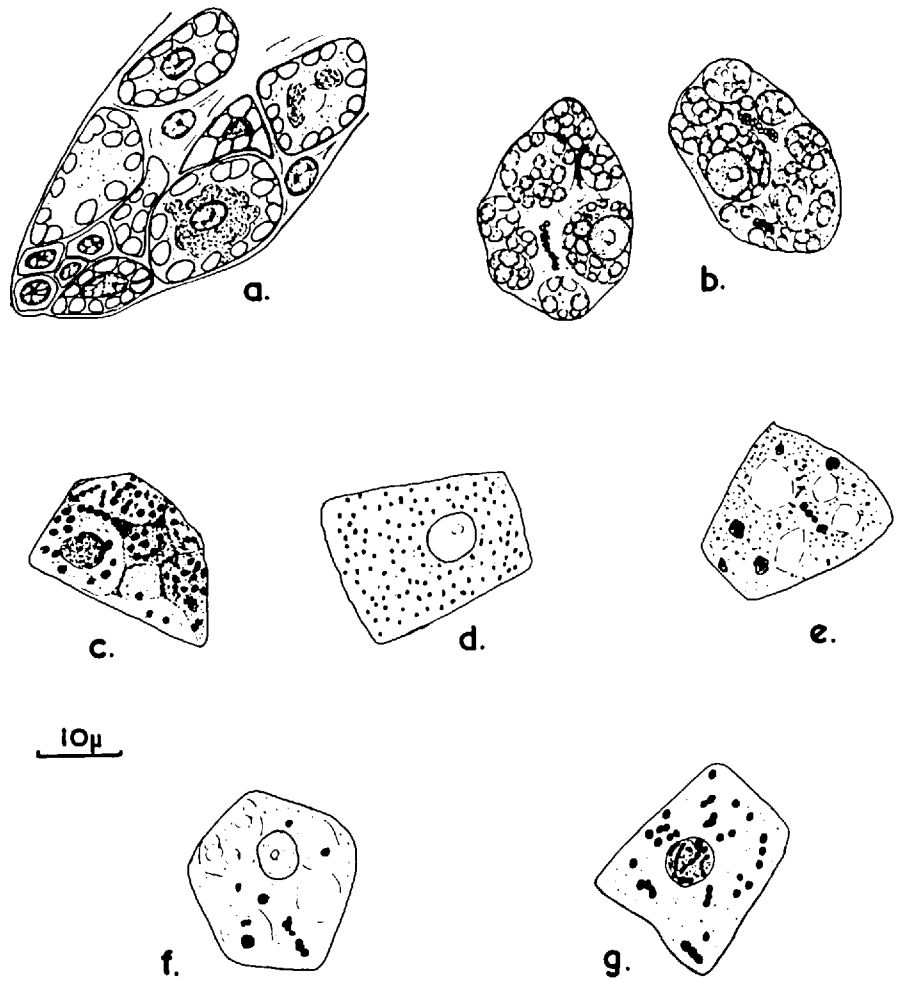


Fig.8 Chemical Constitution of the Vitelline Cells

material often in the form of small granules (Fig. 8c). Protein-free vacuoles were also evident but the organisation of the cell bore little resemblance to that of unfixed material. The protein was rapidly utilised during development.

iii. Carbohydrate.

Large amounts of carbohydrate were present in the vitelline cells of undeveloped eggs. The PAS - positive material was dispersed throughout the cytoplasm in the form of fine granules (Fig. 8d). Quantities of carbohydrate were also present in the cytoplasm of the developing embryo. Glycogen was shown by Best's Carmine procedure to be present in the form of larger granules (Fig. 8e) presumably as a result of the different procedures employed in the two techniques. No acid mucopolysaccharide was detected but fixed material stained with toluidine blue revealed the presence of small rounded purple granules (Fig. 8f) which could possibly be mitochondria.

iv. Lipid.

The fate of lipid components proved to be the most interesting. Staining with Sudan Black B indicated that little lipid was present at the beginning of development (Fig. 8g). The amount detectable on second and successive days was greatly increased. Obviously, a synthesis of lipids was occurring during development. By the sixth day of development lipids predominate in the vitelline cells and are found as large globules throughout the vacuolated cytoplasm. The 'granules' which surround the papilla of the

embryo from the eighth to tenth days of development, are in fact lipid in nature.

The method for phospholipids gave negative results whilst the procedure for neutral and acidic lipids stained the lipid droplets a brown-pink colour (this indicates neutral lipids, i.e. Triglycenides).

From histochemical evidence it is concluded that at the beginning of development the food reserves of the egg are predominantly protein and carbohydrate (glycogen) with small amounts of lipids. At the onset of development a synthesis of lipids occurs so that by the sixth day lipid predominates as the food reserve. From then onwards the lipid is utilised as the major food source so that the fully developed egg contains only small amounts of unused material.

(b) Growth and Weight determinations.

All experiments were performed on eggs between 0 and 10 days of embryonation. The eggs incubated for ten days are recorded as fully developed though in fact they require a further twelve hours to complete embryonation. This procedure simplified manipulation of samples, as fully developed eggs hatch immediately after exposure to light and therefore require special handling techniques.

Daily measurements of embryo size and volume are tabulated below.

Table III.

Day	Length (μ)	Breadth (μ)	Volume (cu.μ)
0	21.8	17.2	3,421
1	24.8	22.3	6,496
2	31.0	24.5	9,737
3	36.4	32.3	20,104
4	40.3	37.6	30,056
5	51.2	41.8	47,015
6	61.1	48.0	74,028
7	95.3	46.0	106,210
8	101.6	44.4	105,309
9	104.0	41.6	95,354
10	106.0	41.8	97,117

The length and breadth of the embryo are plotted against age in Fig. 9a. The volume is plotted against age in Fig. 9b, and finally, to compare the rate of growth on successive days the volume of the embryo is plotted on a logarithmic scale against age in Fig. 9c.

Increase in both length and breadth occurs until the sixth day of development so that the embryo assumes an ovoid shape. This period is one of rapid cell division. From the sixth day onwards, cell differentiation occurs and this is reflected in the rapid elongation. The ratio of length to breadth increases from 1.25 : 1 on the sixth day to 2.5 : 1 on the tenth

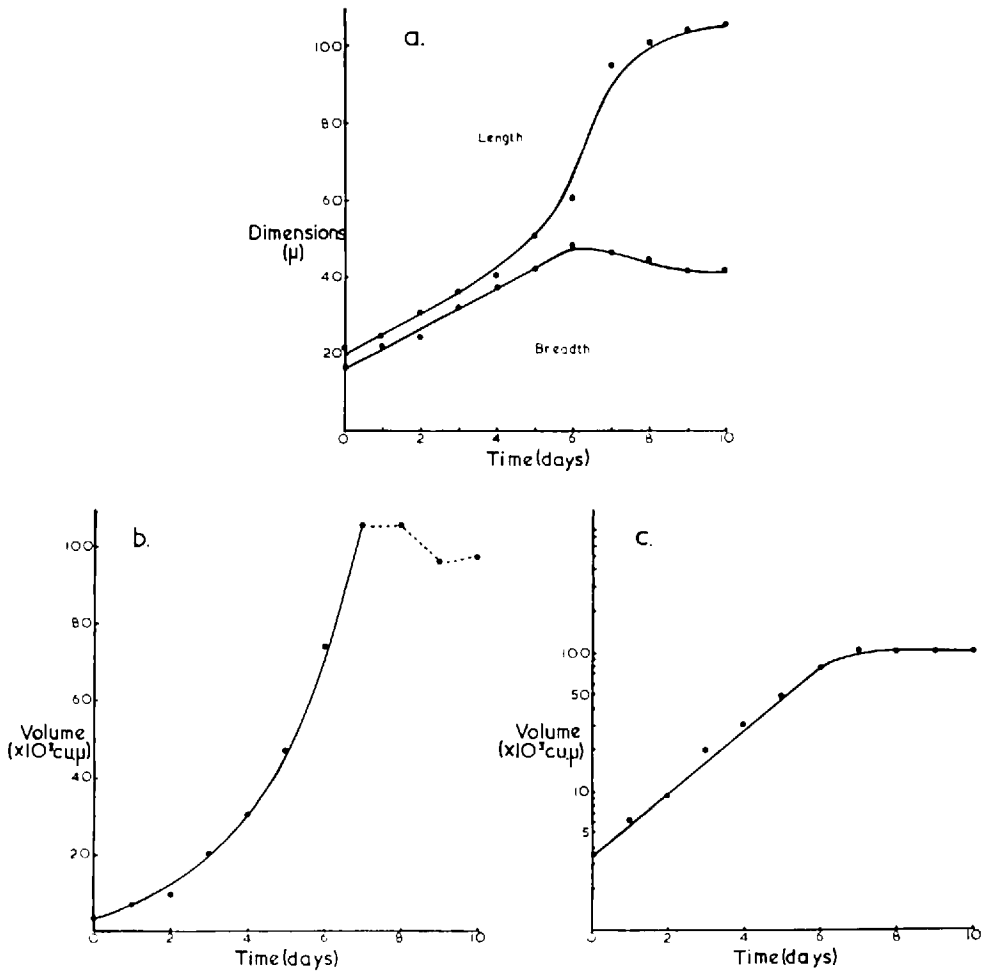


Fig.9 Growth of the Embryo

day. The plot of volume against age is perhaps a better indication of actual growth. The graph is in fact a 'typical' sigmoid growth curve. The transformation of the data to a logarithmic scale shows growth to be a uniform up to the seventh day of embryonation. The reduction of rate of growth beyond this point is attributable to the processes of cell differentiation. The daily growth increment is approximately x 0.8, i.e. the embryo almost doubles in volume per day.

The results of dry and fresh weight determinations of undeveloped eggs, using the glass fibre microbalance, are given below (Table IV). The data are plotted graphically in Figure 10.

Table IV.

No. of eggs	Wet Weight (µg)	Dry Weight (µg)
1106	880	148.5
1107	680	192.5
2069	1440	264.0
2070	1720	280.5
2934	2400	456.5
2935	1640	462.0
3743	3280	583.0
3747	2960	614.0

If the figures for wet weight are truly representative then solids account for only 20% of total weight, rather a low figure compared

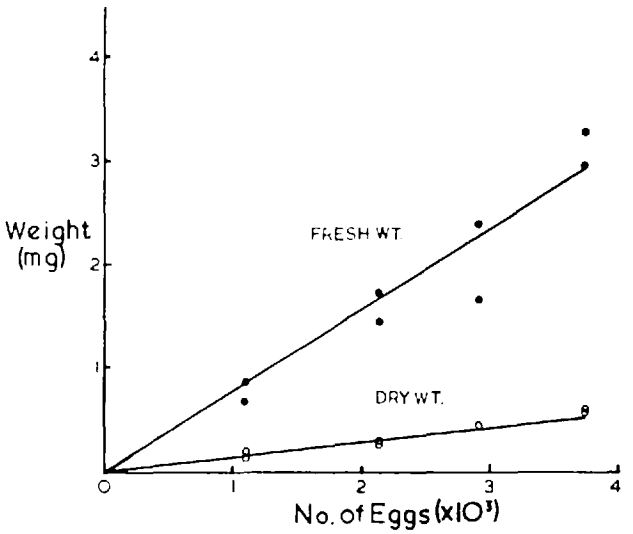


Fig.10a Dry and Fresh Weights

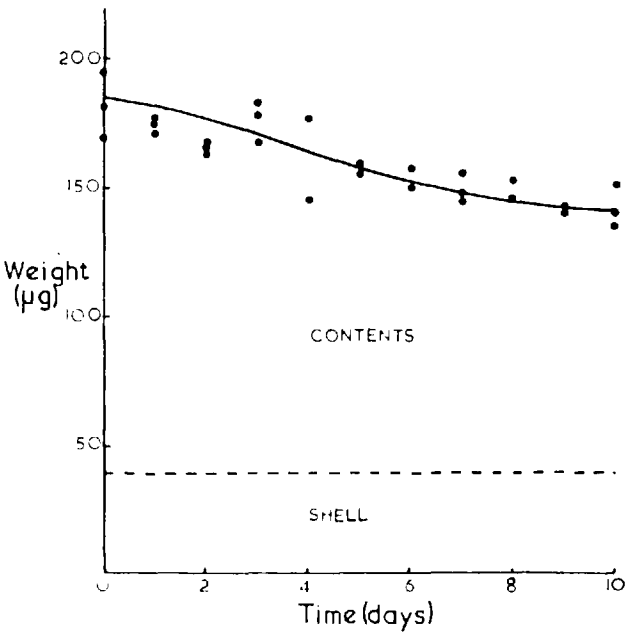


Fig.10b Changes in Dry Weight

to many eggs.

No significant change in fresh weight was detected during development but the dry weight decreased markedly (Table V). The total change from 181.5 µg/1000 eggs to 142.4 µg/1000 eggs represents a decrease of 22%.

Table V.

Day	Weight per 1000 eggs (µg)			Mean
0	180.4	195.1	169.1	181.5
1	174.0	171.0	177.0	174.0
2	168.3	163.7	165.6	165.8
3	178.6	167.2	183.2	176.3
4	145.5	177.7	-	161.6
5	156.5	159.4	-	158.0
6	150.4	158.3	-	154.3
7	148.5	156.4	145.0	149.9
8	153.3	146.8	-	150.0
9	140.5	142.0	-	141.3
10	135.0	140.0	152.3	142.4

The percentage development of the samples was 75.5% so that the true decrease would be greater. Repeat experiments gave variable results and decreases from 175 µg to 128 µg/1000 eggs, and 165 µg to 116 µg/1000 eggs were also recorded.

The results given in Table V are plotted graphically in Fig. 10b.

The curve was fitted by eye and the horizontal line indicates the weight of shell material so that the true change can be assessed (approximately 40% of egg contents are utilised during development). It is evident from the graph that rate of utilisation is greatest during the middle period of embryonation when growth is proceeding rapidly.

The dry weight of shells was determined as 39.1 µg/1000 (mean of seven experiments). The shell therefore accounts for about 20% of the dry weight, quite a high proportion compared to the hen's egg (10%, Needham, 1931).

The dry weight of miracidia was very variable presumably because of the method employed. Estimates ranged from 39.2 µg to 73.0 µg/1000 miracidia with a mean of 61.0 µg (five experiments). Some variation would be expected, particularly with the age of the miracidium, but not of this order of magnitude.

(c) Respiration.

The respiration experiments produced a considerable amount of data which cannot be included here. A sample result (Day 0 of embryonation) is set out in Appendix II together with sample calculations for determining QO_2 and RQ. The rate of oxygen uptake and carbon dioxide evolution is plotted graphically in Fig. 11.

Inspection of Fig. 11 shows that uptake and evolution of gases remains constant for at least $2\frac{1}{2}$ hours. The two-hour result was therefore used to calculate hourly rates of respiration. A summary of the daily

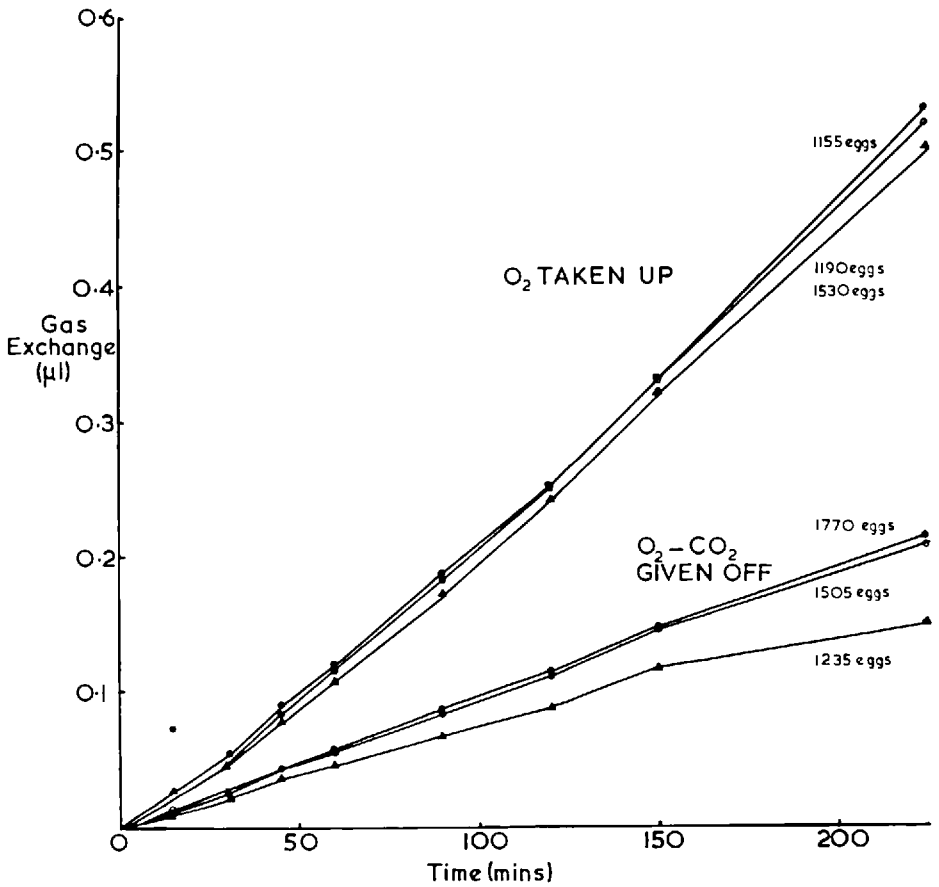


Fig.11 Respiration of Eggs. Day0 Experiment

figures for rates of gas exchange and the calculated respiratory quotients is given in Table VI.

Table VI.

Day	O_2 uptake (µl/hr)				$O_2 - CO_2$ (µl/hr)				R.Q.
	1	2	3	Mean	1	2	3	Mean	
0	0.109	0.106	0.078	0.098	0.032	0.038	0.036	0.035	0.7
1	0.116	0.131	0.108	0.119	0.087	0.065	0.049	0.067	0.48
2	0.122	0.135	0.113	0.123	0.020	0.047	0.035	0.035	0.72
3	0.110	0.105	0.120	0.111	0.041	0.046	-	0.044	0.66
4	0.123	0.145	0.087	0.118	0.012	0.025	0.013	0.017	0.94
5	0.147	0.128	0.087	0.138	0.025	0.022	0.050	0.034	0.83
6	0.096	0.084	0.087	0.090	-	0.020	0.015	0.018	0.88
7	-	0.138	0.147	0.143	0.047	0.060	0.060	0.056	0.64
8	0.200	0.166	0.183	0.183	0.037	0.10	0.064	0.067	0.69
9	0.220	0.216	-	0.218	0.087	0.096	0.108	0.097	0.62
10	0.160	0.218	0.189	0.192	0.073	0.074	0.037	0.061	0.75

The mean data are plotted in Fig. 12a and the Respiratory Quotient values in Fig. 12b.

Inspection of the graph of oxygen consumption and a comparison with Fig. 9a, showing the dimensions of the developing embryo, reveals a similarity of form. The oxygen consumption rises sharply between day 0 and day 1 presumably as a result of activation processes. It is then maintained

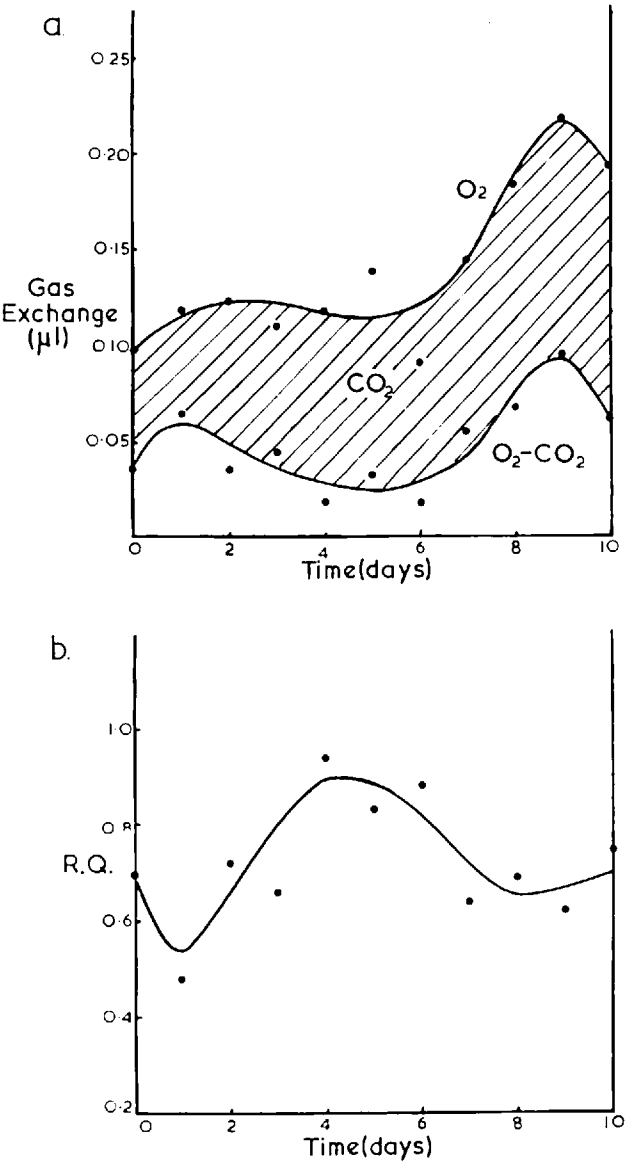


Fig.12 Respiration of Eggs
a. Gas Exchange
b. Respiratory Quotient

at a steady level during the period of embryonic cell division. From the sixth day onwards there is a rapid increase in the rate of oxygen uptake, as cell differentiation proceeds, and finally a slight decrease as development is accomplished.

The carbon dioxide evolution, and hence the Respiratory Quotient, are a reflection of the metabolic changes occurring within the egg, and the type of substrate being utilised as an energy source. For example, carbohydrate would give an R.Q. of 1.0; Protein, 0.8; and Lipid, 0.7. The pattern indicated here is firstly, a close parallel between metabolism and growth as would be expected, and secondly a division of metabolism into three phases. (1) An event occurring at the onset of development which markedly depresses the Respiratory Quotient (0.48 on day 1); (2) A period in which a mixed substrate, predominantly carbohydrate, is utilised; (3) A period at the end of development during which lipid is the main energy source.

In order to express the rate of oxygen consumption in more accurate terms, and to allow a comparison with other work, the QO_2 was calculated. This is defined as the µl of oxygen taken up / mg dry weight of tissue / hour. The figures for dry weight were taken from the previous experiments and corrected to 100% development. The figures for oxygen consumption were treated similarly and the QO_2 was calculated. The results are tabulated below and plotted in Fig. 13a.

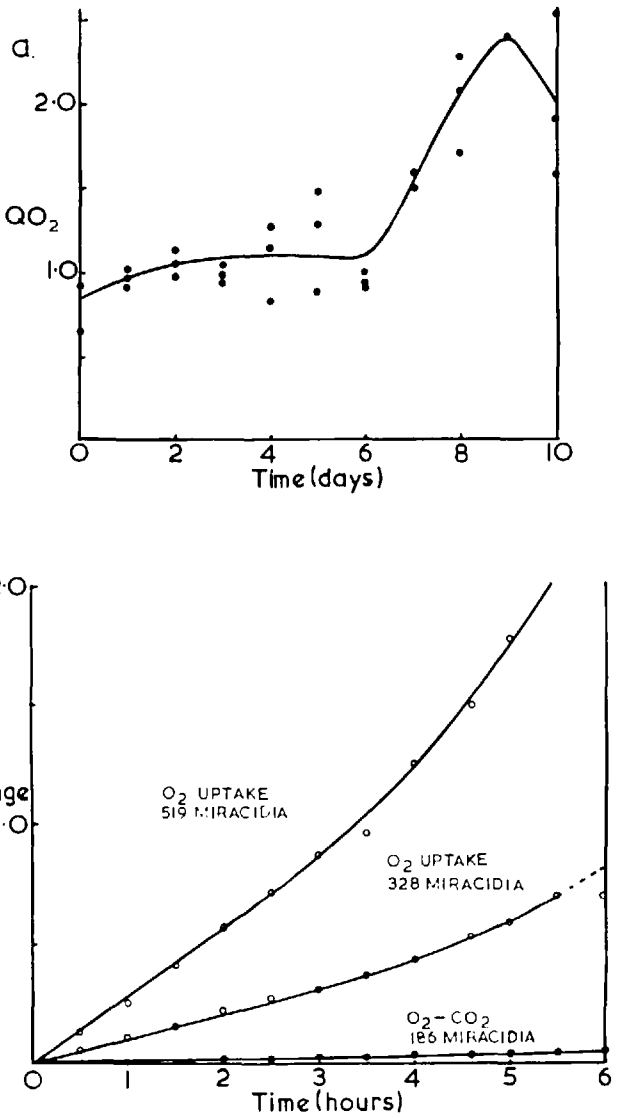


Fig. 13 Respiration
a. QO_2 of developing eggs
b. Gas exchange of miracidia

Table VII.

Day	QO_2			Mean
0	0.93	0.93	0.65	0.84
1	0.97	0.91	1.10	0.99
2	1.05	1.16	0.97	1.06
3	0.97	0.93	1.06	0.99
4	1.17	1.38	0.83	1.13
5	1.47	1.28	0.87	1.21
6	1.00	0.88	0.92	0.93
7	-	1.50	1.60	1.60
8	2.27	1.70	2.08	2.02
9	2.49	2.49	-	2.49
10	1.89	2.58	1.57	2.01

Needham (1931) gives a useful check on the results of dry weight experiments. The total carbon dioxide evolved during development can be calculated from hourly output. 1 µl of carbon dioxide evolved is ~~ to 2 µg of carbon dioxide, and is equivalent to the combustion of 0.76 µg of lipid and 1.4 µg of carbohydrate. The total carbon dioxide produced during development was 52.5 µg (corrected to 100% development gives 78.75 µg of carbon dioxide, equivalent to 30 µg of lipid or 55 µg of carbohydrate/ 1000 eggs). The actual loss in dry weight during development (corrected to 100% development) was 55 µg/1000 eggs so that the figures are in close

agreement.

Experiments on the respiration of miracidia gave more variable results. The QO_2 appeared to be related to the length of incubation after full development. Thus miracidia from eggs incubated for 15 days had a QO_2 of 18.3; 21 days a QO_2 of 9.8; and 23 days a QO_2 of 7.25. The decrease in activity under otherwise uniform conditions must be attributed to the utilisation of reserves during the post-development period of incubation. The QO_2 is approximately double Horstmann's figure and about ten times that of the developing egg.

The Respiratory Quotient of the miracidium was calculated as 0.97 indicating that carbohydrate is the principal source of energy, and this is in agreement with histochemical findings.

The effect of alternating periods of illumination and darkness on the miracidial respiration did not have the expected result. Horstmann has shown that the QO_2 of miracidia declined from 10.3 to 6.1 in about six hours, and that it was lower in darkness than when vessels were illuminated. No such effect was observed here and the rate of oxygen consumption remained constant for 2 - 3 hours after which it increased steadily. Identical results were observed in all four experiments performed and an example is plotted in Fig. 13b. The miracidia removed from respiration vessels were much cytolysed and moribund. The effect of cytolysis on oxygen consumption has been recorded and Needham (1931) quotes Shearer as saying that "whenever you get cytolysis oxygen consumption is of course greatly increased". Cytolysis in the miracidium presumably results from an uncoupling of oxidative

phosphorylation followed by loss of membrane integrity and an oxidation of structural components in an attempt to satisfy energy requirements.

(d) Chemistry of Development.

The variations imposed by technique and material were most apparent in this section. Different batches of eggs gave a range of results for each particular determination. The data given below are therefore selected results, and to enable comparisons to be made, the mean result for each determination was calculated.

i. Protein metabolism.

The results of Total Nitrogen determinations are given in Table VIII (samples of 500 eggs used).

Table VIII.

Day	µg N/500 eggs					Mean
0	5.4	5.7	8.1	6.1	6.8	6.4
2	5.2	5.9	5.8	7.7	6.6	6.2
5	4.8	5.5	6.3	4.8	5.4	5.4
8	3.5	6.7	5.9	4.2	4.6	5.0
10	3.7	4.1	4.1	3.7	4.9	4.1

There is considerable variation between replicates only part of which can be attributed to the technique. The results were subjected to an analysis of variance and were found to be statistically significant at

the 1% level. A repeat experiment gave mean figures of 8.0 µg N/500 undeveloped eggs and 7.2 µg N/500 fully developed eggs. The results corrected to 100% development indicate a loss of 1.6 to 3.0 µg N/500 eggs during embryonation. The figure for total nitrogen multiplied by 6.25 gives the protein equivalent. In this case the protein lost is in the region of 20 - 37 µg/1000 eggs and probably nearer the lower figure. The total protein content of the egg at the beginning of development is approximately 80 - 100 µg.

The nitrogen content of the empty shells was determined as 2.77 µg/500 (mean of seven experiments). This figure is equivalent to 37.5 µg of protein/1000 eggs and is in excellent agreement with the dry weight determination, and indicates that protein is almost certainly the sole constituent of the shell.

The nitrogen content of the miracidium was found to be 1.71 µg N/500 (mean of 5 determinations). Considerable variation was apparent, probably resulting from the method used in preparing the material. The nitrogen content is equivalent to 21.4 µg of protein/1000 miracidia, which is low when compared to the dry weight of 60 µg/1000 miracidia.

The soluble protein content of the undeveloped egg was consistently around 20 µg/1000. The amount decreased during development and a figure of 10.8 to 15.2 µg/1000 eggs (100% development) was recorded on day 10. The decrease could represent either combination into insoluble structural components, or combustion as an energy source.

Electrophoresis of protein extracts was hampered by the poor

protein preparations obtained. The extracts appeared labile, possibly on account of the primitive freeze-drying procedures used, and only one successful extract was prepared. The soluble protein from 7000 eggs was freeze-dried and taken up in 100 µl of water. 20 µl of this solution gave a detectable fraction which migrated 1 cm towards the cathode, in one hour, at pH 8.6. There may have been a second minor component approximately 0.75 cm from the origin.

Confirmation of these results must await better methods of preparation. Soluble protein is, however, definitely present in eggs in quantities sufficient for electrophoretic studies.

Tests for ammonia, urea, and uric acid were all carried out. Of these, only the ammonia test was positive. It was performed daily on aliquots of the incubation medium. Measurements of pH had already shown that this became alkaline during development, and a positive reaction for ammonia indicated the probable cause. It would seem therefore, that ammonia is secreted in small amounts during embryonation, and this in turn implies that ammonia can pass through the supposedly impermeable 'vitelline membrane' complex.

ii. Carbohydrate metabolism.

The total carbohydrate content of undeveloped eggs ranged from 38 to 56 µg/1000. The mean of four experiments was 47 µg/1000. The amount detected at the end of development ranged from 8 to 38 µg/1000 eggs and the higher figure is of course influenced by the percentage development. The

mean figure, corrected to 100% development was 17.7 µg/1000 eggs which gives a loss of 30 µg of carbohydrate during embryonation. Thus, carbohydrate represents 26% of total dry weight before development and only 14% of dry weight at the end of development. These data are in quite good agreement with Horstmann's figures of 32% and 15% respectively.

iii. Lipid metabolism.

The measurement of neutral lipid content at the beginning and end of development supported the histochemical observations. The mean lipid content of undeveloped eggs was 16 µg/1000 and of fully developed eggs 22 µg /1000. The situation was further clarified by measurement of the lipid content of eggs incubated for two days. A mean figure of 10 µg/1000 eggs was recorded. It is not possible to correct this result to 100% development and the figure could be even higher. The mean figure for day 10 corrected to 100% development was 21 µg/1000.

iv. Nucleic acids.

Measurements of the percentage transmission of standard nucleic acid bases, using the Beckman spectrophotometer, did not give a linear result with respect to concentration. A calibration curve was therefore used to determine the concentration of nucleic acid in egg extracts. The method had a sensitivity of 0.5 µg of bases/ml. Undeveloped eggs contained 1.1 µg/1000 eggs and fully developed eggs 1.25 µg/1000 (67% development). The true figure for day 10 would thus be 1.88 µg/1000 and the nucleic acid therefore increases from 0.77% of egg contents at the beginning of develop-

ment to 3.1% in embryonated eggs. These figures are low compared with other material such as bacteria, where nucleic acid often accounts for 10% of dry weight, and could probably be increased by better extraction procedures.

The data recorded in the previous pages are set out diagrammatically in Fig. 14a, b. The situation at the beginning and end of development is recorded in terms of actual quantities/1000 eggs (Fig. 14a), and the percentage change of the same components is given in the second diagram (Fig. 14b). (The areas of the two circles accurately represent the changes in dry weight which occurred).

It can be seen that the loss in dry weight of about 55 µg is accounted for by changes in protein and carbohydrate content. Carbohydrate appears as the main energy source but combustion of protein must contribute something to the overall metabolism. The synthesis of lipid at the beginning of development and its later utilisation is confirmed. The carbohydrate-protein ratio of 3 : 2 can be used to convert the figures for Carbon dioxide evolved into actual material combusted. 78.75 µg of Carbon dioxide are equivalent to 21.3 µg of carbon which if partitioned between carbohydrate and protein gives an equivalent of 54.2 µg of solids utilised during development. This figure is of course affected by such factors as the synthesis of lipid, etc., but is in quite good agreement with total change in dry weight.

Fig. 14a The Chemical Composition of the Egg

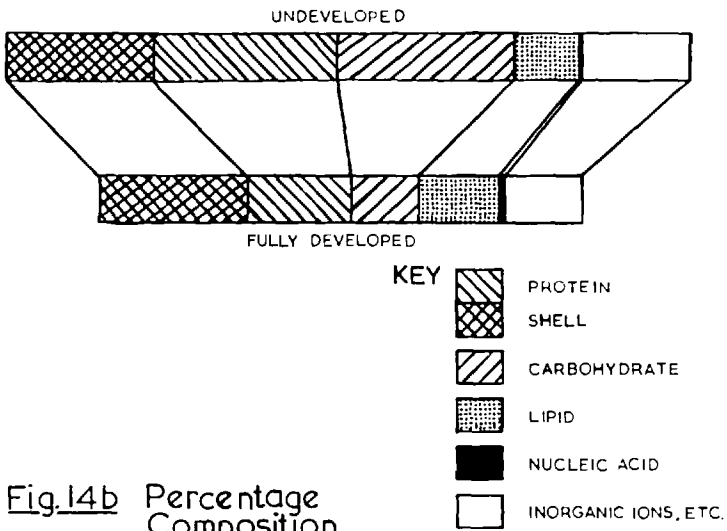
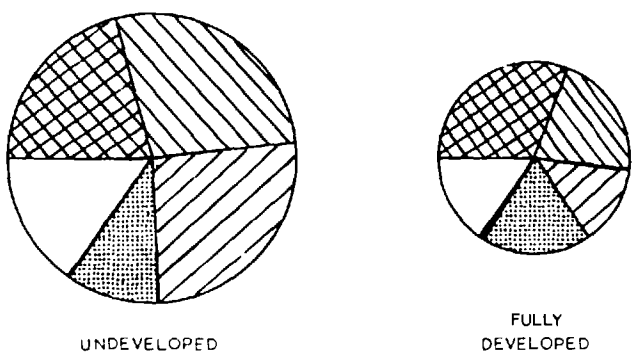


Fig. 14b Percentage Composition



Discussion.

A complete understanding of the biochemistry of embryonation would require many years to attain. A great deal of research has been carried out on many different eggs but the literature is scattered and fragmentary. The findings described here are therefore discussed mainly with reference to "Chemical Embryology" by Needham (1931) covering all aspects of egg metabolism (and still the most important work in the field) and the review of the biochemistry of Ascaris by Fairbairn (1957), which affords a direct comparison with a parasite ovum. The comparison is still a distant one as the biology of the Ascaris ovum differs from that of Fasciola, (e.g., in being an endolecithal egg). It is however the nearest phylogenetic example which has been studied in any detail.

Chemical constitution: The water content of the undeveloped egg (80%) seems a little high compared with the figures quoted by Needham. For fish it is in the region of 50 - 60%; for insects around 65%; for molluscs 50 - 75%; and for Ascaris approximately 70%. The high figure is therefore probably a reflection on the technique used, as the ready plasmolysis of eggs in air accounts for the difficulty in measuring the wet weight. The contribution of the shell to total dry weight is also considerable. The material is solely protein, derived from the vitelline cell globules. It is evident that in the process of shell formation the vitelline cell loses 20% of its solids. Histologically the vitelline globules make up 1/4 to 1/5 of the total vitelline cell volume and a figure

of 20% by weight is therefore accurate. The remaining 80% of undeveloped egg solids have been shown by histochemical techniques and direct chemical analysis to consist of large amounts of protein (27%) and carbohydrate (26%), and small quantities of lipid (10%). The egg of Fasciola seems unique in this respect. According to Fairbairn, Ascaris ova contain protein (41%, part of which is shell material), carbohydrate (16%), and lipid (35%). Carbohydrate, possibly on account of its low energy to volume ratio, generally plays little role in embryonation. Molluscs alone have carbohydrate as an important energy source and there it is stored in the form of the unusual polysaccharide, galactogen. The more common arrangement in animals as distinct phylogenetically as birds, fish, and insects is an energy store of part protein and part lipid. The possible significance of the findings is discussed later.

The Physiology of Embryonation: The development of the embryo both histologically and biochemically can be divided into two periods. These are best illustrated by changes in the length and breadth relationships of the embryo. The first phase of growth is a period of rapid cell division producing an ovoid ball of undifferentiated cells. The second phase from day 6 onwards consists of the period of cell differentiation and increase in length of the embryo. The two phases are also illustrated by changes in the rate of oxygen consumption of the egg (see Fig. 13a).

The values obtained for daily changes in dry weight are useful only in that they provide totals against which other work can be compared.

Approximately 38% of egg contonts are combusted during development. Needham quotes the hen's egg as losing 18% and the silkworm 17% of total dry solid during the same period. The efficiency of the metabolic processes is not therefore high and the material included in the embryo accounts for only 75% of egg contents at the end of development. The remaining 25% is represented by the viscous cushion, sacs, lipid droplets, etc. Judging from the volume occupied by the viscous cushion, its weight must account for a significant part. The figure for total solids lost during development was confirmed by the value calculated from total carbon dioxide evolution.

The process of embryonation in the fluke egg is obligatorily aerobic and under natural conditions the oxygen tension of the environment may be low. Rowcliffe and Ollerenshaw (1960) noted that eggs trapped in faeces did not embryonate.

The results of the respiration experiment are interesting. The calculation of QO_2 allows a direct comparison to be made with Horstmann's figures. The values of 0.8 - 0.9 obtained here for the first phase of growth are about three times those of Horstmann and the figures of 2.0 to 2.5 in the later phase are approximately double his values. The configuration of the curve given in his paper is also different. One rather puzzling feature of his work is the use of buffered 0.9% saline as a culture medium. The natural environment of fluke eggs is fresh water and in the experiments recorded here distilled water (pH 5 - 7) was used. The fact that Horstmann used buffered 0.9% saline (pH 7.4) may account for the disparity in results obtained. In Ascaris ova the maximum QO_2 observed by

Passey and Fairbairn (1955) during the period of embryonation, was 2.7 at 30°C.

The presence of an active oxidative metabolism in the developing ovum implies the participation of mitochondria. These are known to be present in some numbers in the fully developed miracidium, and histology also indicates that they may be present in vitelline cells.

The calculated values of respiratory quotients give some clue to the substrates utilised. The reason for the decline of the quotient to 0.48 on the first day of development, is not immediately apparent. It is quite possibly connected with the synthesis of lipid found to occur around this period. The process must in some way involve carbon dioxide. The quotient rises from this low level to a peak of 0.94 on day 4 (mean value 0.81 for days 2 - 6) and this indicates a mixed substrate with carbohydrate predominating. Over the remainder of development there is a period of increased oxygen consumption and decreased respiratory quotient. There is a mean value of 0.68 for days 7 to 10 indicating a predominantly lipid substrate. It must be borne in mind that factors other than direct combustion can affect the respiratory quotient. Indeed, the events on the first day of development indicate such a feature here. Synthesis, and interconversion of energy sources are the main causes of a distorted RQ reading.

The Metabolism of the Egg: Experiments measuring decreases in particular components indicated that overall, carbohydrate and protein were the main

energy sources. The situation is complicated by the synthesis of lipid at the beginning of development but the source of this has not been identified. It is also apparent from the results of histochemistry and respiration studies that lipid is utilised as an energy source during the latter part of development. The environment of the egg is said by Needham to be the determining factor in the type of food reserve stored and utilised. In general aquatic eggs with facilities for nitrogenous excretion utilise more protein (62% of total) than terrestrial eggs (6.8% of total). On the other hand, terrestrial eggs utilise more lipid (81% of total) than aquatic eggs (32% of total).

Neither Fasciola nor Ascaris follow this general pattern and the trends in these two are almost diametrically opposite. In Ascaris carbohydrate is utilised during early development, followed by lipid, much of which is converted into carbohydrate (cf above for Fasciola). The situation in the fluke egg may be connected with its unique structure, and origins of the vitelline cells. If these are, as Hyman suggests (see Part II), transformed ova then this explains the high initial carbohydrate content. It is possible that the lipid synthesis is a form of late maturation. On the other hand it could in some way be connected with the effects produced by the formation of the vitelline membrane in the mid period of development.

The detection of ammonia in the incubation medium is consistent with the utilisation of protein as an energy source. Needham quotes similar findings in echinoderm, fish, and amphibian eggs.

Very little is known about the dynamics of the embryo/vitelline

cell system. Most workers have described it as a degeneration of yolk cells, with subsequent utilisation of the disordered mass by the embryo. This does not appear to be the case here and the actual synthesis of lipids plus the possible presence of mitochondria point to an active metabolism on the part of the vitelline cells. The food reserves are presumably degraded to glucose, amino acid or acetyl CoA, in which state they could be taken up by the embryo. The membrane of the vitelline cells may allow simple diffusion to occur, since its ready permeability to water and rapid swelling can readily be demonstrated by squashing an undeveloped egg. The only evidence at present for hydrolytic mechanisms is the fact that nuclei and cytoplasmic constituents are broken down; an activity usually controlled by lysosomes or similar particles. Horstmann concluded that glycogen was taken up in the macromolecular state but quoted unpublished work as evidence. The supposition seems unlikely as transport of macromolecules is not a common occurrence, except by pinocytosis, and no pinocytic vesicles were observed in electron micrographs of the miracidial epidermal cells. Evidence for the active uptake of glucose was put forward by Bryant and Williams (1962). They used ^{14}C-labelled glucose and later detected glycolytic intermediates in the miracidium. They also noted that the presence of saline and glucose both increased the survival time of the miracidium. It is quite possible that mechanisms for active transport occur within the developing embryo.

In conclusion it may be said that the egg of Fasciola, whilst having many points of similarity with other freshwater eggs, has several

unique metabolic features. These are no doubt related to the unusual structure of the egg and development appears to be a dynamic balance between the embryo and surrounding vitelline cells. As to the metabolism of the free miracidium, little can be said. It is a more active organism than the developing embryo, and its main energy source appears to be carbohydrate.

PART V. THE STRUCTURE AND FUNCTION OF THE FLAME CELL SYSTEM

Introduction.

Protonephridia, or flame-cell systems are a characteristic feature of several invertebrate groups and they are particularly well developed in both freshwater and parasitic platyhelminths. Most digenetic trematode miracidia possess one or two pairs of flame cells and associated ducts. The general structure of the system in the embryo and miracidium of *Fasciola* was described in Part II.

The fine structure of the flame cell was described by Kümmel (1958, 1959). It consists of a large nucleus surrounded by cytoplasm, and a cylindrical outgrowth of this encloses the aggregation of cilia which constitute the flame. The cylinder is corrugated on both inner and outer surfaces and was thought by Kümmel to be the site of filtration. At its distal end the cylinder narrows to form the excretory duct.

Pantelouris and Threadgold (1963) have described the fine structure of the excretory system of the adult *Fasciola hepatica*, and Pedersen (1961) has described the protonephridia of a freshwater Planarian. Pantelouris and Threadgold noted the presence of numerous vesicles and microvilli in the cells of the excretory ducts, and attributed to them a secretory function. Available evidence indicates a close similarity of fine structure between the miracidial system and those of the adult *Fasciola* and the Planarian.

The function of the flame cell system has been variously interpreted. Usually, however, an excretory or osmoregulatory role is ascribed to it.

The evidence is very slender and the subject was reviewed by Martin (1958). He states that "the cilia presumably set up a current in the tube..... (and) one has to assume either that the flame is giving rise to a filtration pressure, or that it is necessary for mixing of a secreted fluid, or that it has no known function". Indeed the only positive evidence is the fact that in organisms such as rotifers or cercariae, possessing a bladder, the rate of pulsation of the bladder is said to be inversely related to the osmotic pressure of the medium.

It is probable, considering the varied environments in which animals possessing flame cells are found, that several functions can be ascribed to the system.

The flame cell system in the miracidium of Fasciola is of particular interest because during the hatching process the rate of 'flame' activity increases rapidly. In the miracidium of Schistosoma haematobium this has been attributed to an increase in osmotic work performed (Reisinger, 1923).

A study of the variations of flame cell activity during development, hatching, and free life, was carried out. Attempts were made to analyse the mechanism of ciliary control and to determine the function of the system.

Methods.

The individual cilia of the flame cell exhibit an undulatory type of activity and probably due to the proximity of the basal bodies, beat in unison rather than metachronally. This means that the characteristics of

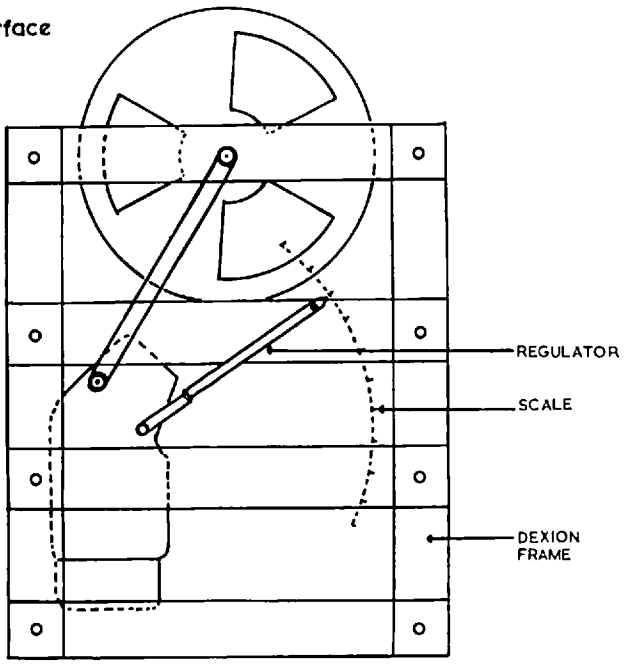
each cilium are reflected in the movements of the whole aggregation or flame. In an analysis of activity the two may be considered synonymous.

Wavelength, amplitude, and frequency are the three variables of ciliary movement. In the confined space provided by the cytoplasm of the flame cell, it is difficult for amplitude and wavelength to vary greatly and frequency undergoes the most apparent changes. Methods of measuring the flame cell frequency were therefore investigated with the necessary restriction that the method selected should be a simple and quickly assembled device.

Gray (1930) reviewed the common methods of measuring ciliary activity, which are usually high speed photography or stroboscopy. Photography of the flame cells was found to be impossible because of the intense illumination involved. All but the lowest light intensities were found to inhibit hatching and to depress flame cell activity. A stroboscope designed to interrupt the microscope illumination was constructed. (The plans are given in Fig. 15). A variable speed gramophone motor was used as the power source, and by suitable gearing would rotate the disc at 60 - 450 r.p.m. The removal of three sectors in the disc gave the stroboscope a 'flash rate' of 180 - 1350/minute. Unfortunately, this method also failed because the flashing light excited both embryos and miracidia. These moved around in the egg rendering measurement impossible. Direct methods therefore seemed out of the question and comparative methods were devised. These included visual matching of flame cell activity against a pulsating light, and against the condensed sine curve of an oscilloscope. The pulsating light and oscilloscope image were superimposed on the microscope field using a camera

TOP VIEW

(Hardboard surface removed)



FRONT VIEW

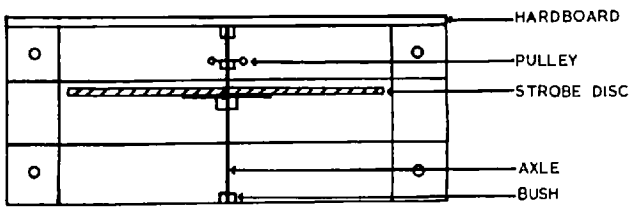


Fig.15 Plans of Stroboscope

lucida. An audible comparison method incorporating an electronic metronome (French, 1961) was also tested and was found to be superior. The variable frequency signals from the electronic metronome were relayed to the operator via a pair of earphones and correlated with the visual undulations of the flame cell cilia. The unit was calibrated against a Muirhead Decade Oscillator and gave consistent readings.

Using this device a study was made of variations in flame cell activity during development and hatching. The effect of osmotic pressure and temperature on the flame cell activity of the miracidium was also measured. Further details are given in the text.

Results.

The flame appearance, according to Kümmel, results from the shorter length of the outer cilia in the aggregation. The wavelength and amplitude of the flame were determined as 2.0 μ and 0.4 μ respectively. Using the electronic metronome variations in frequency from 1 - 14 beats/second were recorded. With practice it was possible to measure flame cell frequency with the following accuracies:

0 - 4 beats/sec. acc. of 0.1 b/sec.

4 - 10 " " " " 1.0 b/sec.

10 - 14 " " approximate beat.

The method was particularly uncomfortable for the operator and the metronome could only be used for short intervals followed by rest periods.

Rate of beat during embryonation.

The flame cells can first be discerned on the seventh day of embryonation. They are not fully formed and the cilia undergo rapid vibrating movements. As the cells develop, this activity is translated into the characteristic undulations of the flame cells. There is at the same time a reduction in the frequency of beat so that when the egg is fully developed the 'flame' is almost stationary.

The activity of the flame cell cilia of embryos at different stages of development was recorded, together with length of the embryo. A heat filter was interposed between the light source and microscope condenser and all measurements were carried out at 20°C. The data from seventy determinations is summarised in Table IX and the actual figures are plotted graphically with the summarised data superimposed in the form of a histogram (Fig. 16a).

Table IX.

Size of Embryo	Mean Frequency
60 - 69 μ	6.0 b/sec.
70 - 79 μ	6.3 "
80 - 89 μ	6.2 "
90 - 99 μ	4.4 "
100 - 109 μ	3.2 "
110 + μ	2.8 "

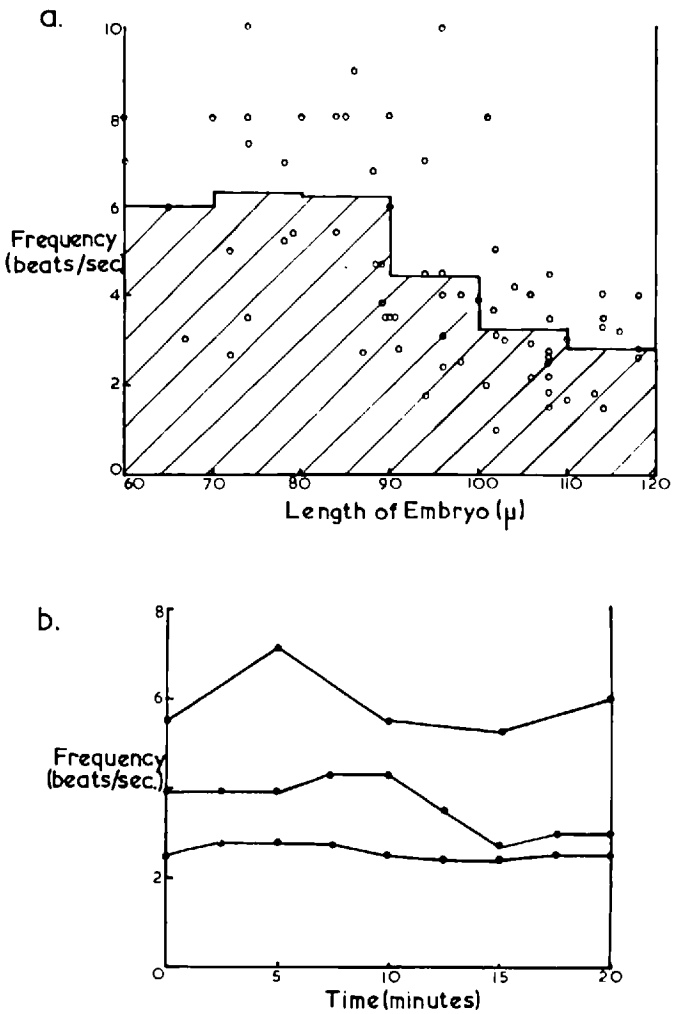


Fig. 16 Flame Cell Activity
a. during development
b. of a single flame cell

The reduction in frequency during development is confirmed. The variations in frequency of beat of single flame cells over short periods, was also recorded and sample results are plotted in Fig. 16b.

Rate of beat during hatching.

Exposure of fully developed eggs to light results in stimulation of the miracidia and is followed after a short interval by hatching. During this period after stimulation the flame cell activity increases rapidly, and attempts were made to record the changes. This entailed transferring eggs from culture vessels to the microscope in complete darkness, quickly finding a suitable specimen and commencing to take readings. There was often a delay of 2 - 3 minutes before a suitably orientated egg was found and initial readings could therefore not be taken. It was presumed, however, that the flame cells were beating at the frequency of 2 - 3 per second recorded immediately previous to maturity. Light of the lowest intensities had some inhibiting effects and great difficulty was encountered in measuring the changes. In order to do this, the heat filter was removed and the corresponding temperature increases raised the threshold at which inhibition would occur.

A typical 'hatching curve' is shown in Fig. 17a. Activation of the flame cell system is very rapid and a plateau of maximum activity was attained 2 - 4 minutes before hatching. Once this plateau of 12 - 14 beats /second had been reached and maintained for a short time, inhibition could no longer be induced. Inhibition of flame cells in miracidia undergoing

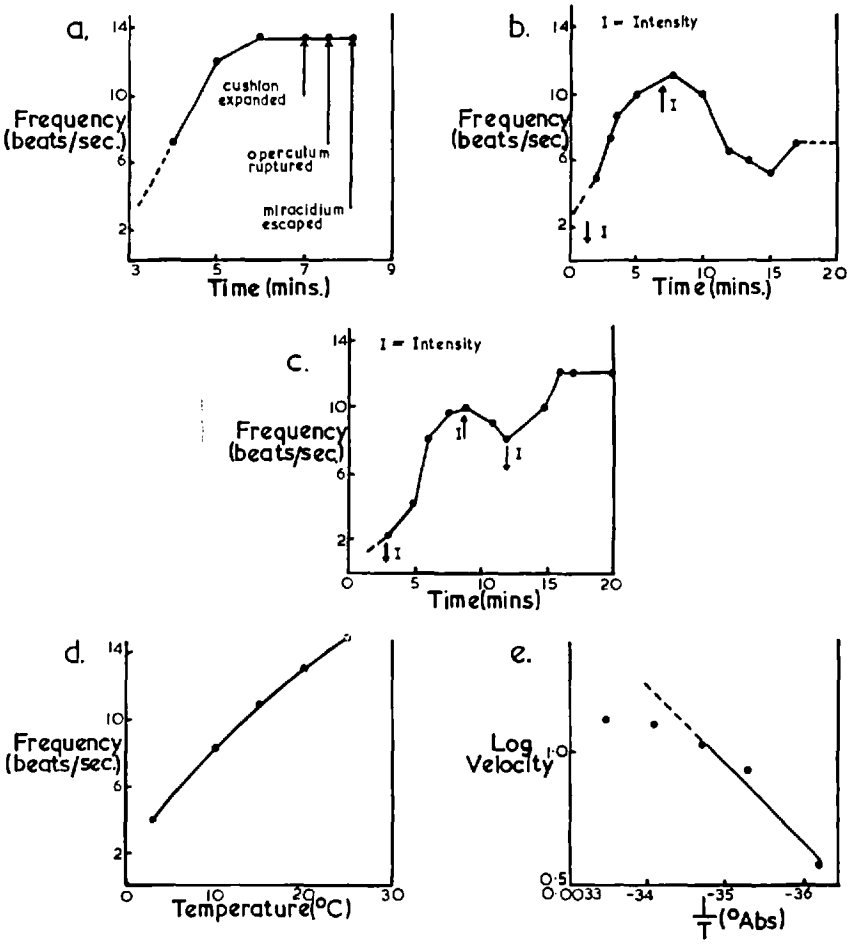


Fig.17 Flame Cell Activity
a. hatching curve
b. inhibition by light
c. inhibition followed by hatching
d. effect of temperature
e. Arrhenius plot of temperature data

activation could be brought about simply by raising the microscope condenser and thus increasing light intensity. The rate of beat of the flame cells decreases to a lower level and will remain there until light intensity is reduced (Fig. 17b). When this is reduced, the activity again increases to the high plateau level and hatching occurs shortly afterwards (Fig. 17c).

Effect of temperature on frequency of miracidial flame cell activity.

It appeared that the shell would provide a physical and chemical barrier to osmotic and temperature variations. The effect of these features was therefore assessed using the free miracidium. Observations on living specimens were facilitated by trapping them in the meshes of a lens-cleaning tissue and examining immediately at X 1000 magnification. In the temperature experiments miracidia were hatched at 20°C. and then placed, in the dark, in the appropriate constant temperature room, to equilibrate for ten minutes. The miracidia were then prepared for observations and flame cell activity was measured. Trapped miracidia rapidly became moribund and measurements were therefore taken only during the first two minutes of observation. The results obtained are summarised below and plotted graphically in Fig. 17d.

Table X.

Temp. (°C.)	Frequency (beats/sec.)	
	Range	Mean
3	2.9 - 4.8	3.9
10	6.5 -10.0	8.25
15	9.0 -12.0	10.8
20	12.0 - +14	13.2
25	+14	+ 14

The frequency at 25°C. was so high that the flame cells appeared to be stationary (a stroboscopic effect) and were difficult to detect. From the figures it is possible to calculate the Arrhenius temperature characteristics for the frequency of flame activity (Sleigh, 1956). The Arrhenius equation describes the effect of temperature on the velocity of a process and can be stated as

$$\log K = C - \frac{\mu}{RT}$$

where K is the velocity of the process;

μ R and C are constants;

T is the absolute temperature.

Agreement with this equation will be indicated if a straight line is obtained when log K is plotted against the reciprocal of Absolute temperature (Fig. 17e). There are insufficient data in Fig. 17e to allow firm conclusions to be drawn. It is apparent, however, that with increasing temperature the linearity is not maintained. There could be two explanations for this, firstly that it is a reflection on the accuracy of the measuring technique, and secondly that there is some inherent feature of the ciliary function which is not capable of responding to increases in temperature above a certain level. Extrapolation of the line to 30°C. would mean a beat of 25 - 30 per second, an improbable level. The optimum temperature for embryonation and other processes in free living stages of flukes is known to be about 26°C. (Rowcliffe and Ollerenshaw, 1960). It may be therefore, that 'failure' of the flame cell system above these temperatures is a

contributory factor to the level of the optimum temperature.

Effect of Osmotic Pressure.

The osmotic pressure of the external medium was varied by using a series of salines (from 0.2 - 1.0%). Eggs were allowed to hatch in these solutions and miracidia were then mounted and observed. All experiments were carried out at 20°C. and the results are summarised below.

Table XI.

Concentration of Saline	Frequency (beats/second)	
	Range	Mean
0	12.0 - + 14	13.2
0.2%	11.0 - 14.0	12.3
0.5%	10.0 - 14.0	11.9
0.8%	10.0 - 14.0	11.8
1.0%	Eggs failed to hatch	

There was no significant difference between miracidia in distilled water and 0.8% saline. Eggs in 1% saline failed to hatch and exhibited abnormal flame cell behaviour. In these eggs the general pattern was for the flame cells of the miracidium to attain a normal beat of 12 - 14/second. Immediately the viscous cushion expanded the flame cell beat was depressed, in some cases to $>$ 1/second. The operculum did not rupture in these circumstances because of insufficient internal pressure. Some weakening of the

operculum must, however, have occurred because it was possible to rupture the operculum by application of external pressure. The miracidium was not expelled but flame cell activity increased again to about 10 beats/second. The miracidium rapidly became moribund. It must therefore be concluded that the excessive compression of the miracidium caused by the great expansion of the cushion in 1% saline results in an impairment of flame cell activity. Removal of the compression allows flame frequency to revert to normal behaviour.

Observations on the system using vital dyes.

Neutral red and Leuco-methylene blue were used to aid observations on the miracidium (Part II) and several interesting points were noted. Leuco-methylene blue had a differential effect on the miracidium, first staining external regions and the large, posterior, triangular cells. Next, epidermal cilia were paralysed and the posterior region of the miracidium contracted. (The paralysis may have been connected with energy supplies to the miracidium since the mitochondria of the epidermal cell stained strongly). Finally, the dye penetrated to deeper tissues and flame cells ceased their activity. The contraction of the posterior regions of the miracidium occluded the excretory pores. This was quickly followed by an enormous expansion in duct diameter which was maintained until the flame cells were paralysed by the dye. On cessation of flame activity the ducts immediately collapsed. It would therefore seem that the activity of the flame cells results in the production of a hydrostatic pressure within the ducts. The reduction in tubule diameter has been observed in normal miracidia when flame cell activity ceases and probably accounts for the difficulty in locating the tubules in fixed material.

When Neutral red was used the fluid in the tubules appeared pinkish in colour. It is possible that the flame cell system was at some point permeable to the dye. The granules surrounding the most distal portion of the tubule had a particularly marked affinity for the dye which could indicate secretion or uptake at that site.

In saline the tubule diameter did not show the expected decrease. It was if anything slightly greater than in distilled water. The mode of action of the system would therefore seem to be more complex than previously postulated.

Discussion.

The purpose of the work was to discover something of the function of the flame cell system and its regulation. The microscopic dimensions do not allow conventional analysis techniques to be employed so that any assessment of function must be indirect. It is possible that conventional techniques could be applied to mature flukes but the different environment must have some modifying effect on the primary function, whatever this may be.

The ciliary activity of the flame cell is of the undulatory type. Strictly speaking this term is applied only to a flagellum, but Sleigh (1962) considers the distinction between cilium and flagellum to have only functional significance.

The variations in activity give some information concerning the method of control. The overall reduction in rate as the flame cell develops coincides with the appearance of the characteristic rhythmic undulatory activity.

Gray (1932) put forward the concept that the cilium can be likened to cardiac muscle. Once a rhythm is established it becomes an integral part of the function and requires no external reinforcement. Control of ciliary activity is brought about in almost all cases investigated, by inhibition of this basic rhythm. The suppression of activity as the embryo develops probably indicates the occurrence of a similar kind of inhibition here. It seems to occur gradually, but this cannot be taken as evidence for a chemical rather than nervous origin. The epidermal cilia are at this time either quiescent or show slow metachronal rhythm. Clearly therefore, they must be affected by a separate control mechanism.

Exposure of the fully developed miracidium to light results in activation of both flame cells and epidermal cilia. Once maximum flame cell activity has been attained it cannot be modified in any way by external stimuli other than drastic treatments resulting in death of the miracidium. It had been hoped to correlate the increase in beat of the flame cells with increasing osmotic work since this was how the response had been previously interpreted (Reisinger, 1923). It was found, however, that increased osmotic pressure of the external medium had no modifying effect on flame cell activity. It was also discovered that high light intensity during the hatching process would inhibit the rate of flame cell beat. It is clear, therefore, that the phenomenon at hatching is an activation process having no correlation with a decrease in osmotic pressure of the egg contents.

The process could be caused either by direct stimulation, or by removal of an inhibitor. The suppression of beat which occurs during devel-

opment implies the latter. What is certain is that a primary or secondary connection with the nervous system must exist since the light receptor organs are an integral part of this.

No connection between the nervous system and flame cells was observed. Application of one of the many tests for neurosecretory substances. (The Paraldehyde-Fuchsin test of Gomori, 1950, modified by Halmi, 1952) to sections and whole mounts of miracidia gave a positive reaction in the posterior triangular cells and primitive gut. Unfortunately Gomori states that the reaction will stain elastic fibres, gastric mucosa, and some mucins as well as neurosecretory substances. The criterion of a neurosecretory cell must therefore remain as a P.A.F. positive cell having a direct connection with the central nervous system, and no such connection was observed here.

The effect of temperature on the function of the system was not unexpected. The non-linearity of the relationship to the Arrhenius equation implies an upper limit to the function of the flame cells which must impose further limitations on the organism as a whole.

Variations of external osmotic pressure of the environment did not modify flame cell activity until toxic levels were reached. It is clear, therefore, that if the flame cell system is concerned with osmoregulation then the actual regulation is not controlled by variations in flame cell activity. In work on the cysticercus of Taenia crassiceps (Wilson, 1962) it was found that the flame cell system was active and well developed but the cysticercus possessed little powers of osmoregulation. On the other hand, Pantin (1947) has directly correlated flame cell activity with the degree of hydration of

the organism, in a terrestrial nemertine Geonemertes.

The variations in tubule diameter, noted in the miracidium, indicate that the flame cell is responsible for the production of a hydrostatic pressure within the ducts, and that the duct walls are not readily permeable to the fluid in the lumen. These facts agree with Kümmel's interpretation of the flame cell as the site of filtration. The final composition of the fluid excreted is probably determined in the tubule region. Vesicles were noted by Pantelouris and Threadgold, and Pedersen, in the surface layers of cells forming the tubules. Pedersen considers the cells to be similar to the convoluted tubules of mammalian kidney. Danielli and Pantin (1950) have also described the presence of alkaline phosphatase in the tubule cells of a triclad and a nemertine. This is of interest because alkaline phosphatase seems to be associated in some way with the sites of active sugar uptake (e.g. intestinal mucosa), though its exact role is unknown. All these facts point to the tubule region as a possible site of selective reabsorption of lumen contents, either by pinocytosis and/or active transport.

It is difficult to see how the problem could be resolved in the miracidium since conventional techniques are out of the question. Indirect evidence could possibly be obtained by the use of radio-active isotopes.

It is possible to put forward a hypothesis concerning the mode of action of the flame cell system. The cilia appear to possess an inherent activity similar to that of cardiac muscle and modifiable by superimposed stimulation or suppression. The activity of the 'flame' results in the production of a hydrostatic pressure in the tubule. This occurs through the

continual movement of fluid from base to tip of the cilia and in turn results in a reduced pressure around the base of the flame. This is the probable region of filtration through the cytoplasmic corrugations described by Kümmel. As the fluid passes down the tubule, selective reabsorption probably occurs so that a true excretory fluid is produced. Osmoregulatory control could be brought about by a construction at some point in the system. An increased tubule pressure would then result and the efficiency of the flame cell would be correspondingly reduced, less fluid therefore passing into the tubule lumen. It must be emphasised that this is only a hypothesis and its validation presents a problem for the future.

PART VI. THE HATCHING MECHANISM.

Introduction.

Speculation on the hatching mechanism of digene eggs has been considerable and three main interpretations have been put forward. Thomas (1883) suggested that the mechanical exertions of the miracidium were responsible for rupture of the operculum. Barlow (1925) favoured the presence of a hatching enzyme which dissolved the opercular bond. Mattes (1926), on the other hand, considered that an increase in internal osmotic pressure was responsible for the rupture of the opercular bond and postulated that the expansion of the cushion prior to hatching was in some way connected with the production of such a pressure.

Rowan (1956, 1957) intensively reinvestigated the hatching mechanism in the egg of Fasciola, and his findings are now frequently quoted as a true interpretation of the mechanism. Briefly, these were as follows: exposure to light induces the miracidium to release a hatching enzyme which dissolves the opercular bond. This enzyme acts from within the shell only, and its effect can be simulated by the application of trypsin and pepsin. It was therefore considered to be proteolytic in nature. The viscous cushion plays a passive role in hatching and was thought to be colloid-like or at least part protein. Its expansion at hatching was interpreted as a change of state from contracted gel to expanded sol. Evidence was presented to show that the expansion of the cushion is accompanied by an "exosmosis" of salts from the egg, presumably caused by damage to the vitelline membrane by the hatching

enzyme. The expulsion of the miracidium was considered to be due to the hypertonicity of the egg contents.

As stated in Part II some of Rowan's experiments are open to criticism, particularly the one concerning "exosmosis" of salts. (This should more correctly be termed diffusion). He exposed developed eggs to a temperature of 65°C. for ten minutes, observed that the viscous cushion had expanded, and by a capillary vapour pressure method proved that salts had diffused out of the egg. He therefore concluded that the exosmosis of salts is associated with, and perhaps accounts for, the expansion of the cushion. The criticism here is that a temperature of 65°C. would destroy the integrity of most biological material leading to diffusion outwards of contents, and this is not a priori, a sufficient reason for believing that the same occurs under natural conditions.

A second criticism concerns the fact that a proteolytic hatching enzyme, to reach the operculum would have to pass through or round the viscous cushion. This seems an unlikely occurrence as Rowan himself demonstrated that proteolytic enzymes rapidly destroy the cushion.

A third point concerns the pressure experiments he performed. It must be emphasised that the effect of external pressure from one direction only upon a spheroid, must have quite different results from the production of an internal pressure in such a system.

Lastly, the viscous cushion, thought by Rowan to be homogeneous throughout, was shown in Part II to be fibrillar in nature.

It was clear, therefore, that a reappraisal of the events bringing

about hatching was necessary, if a true understanding of the phenomenon was to be attained. A section of the work on the chemistry of the viscous cushion was submitted as a dissertation in part fulfillment of the requirements for the Diploma of Chelsea College, in Biochemistry. The methods and results are relevant here and the work is therefore included in a summarised form.

Methods.

(a) General Observations.

The events occurring in the egg during hatching were carefully observed, and recorded photographically. Further simple experiments were then carried out in an attempt to analyse each step in the hatching mechanism and to place the observations on a quantitative basis. The structure and chemistry of the cushion were then investigated.

(b) The Stimulation of the Miracidium.

Simple experiments were performed to test the effect of light on rate and number of miracidia hatching. All experiments were performed in a light-proof C.T. room at 20°C. The experimental vessel used was a one-inch diameter tube. A light source of variable intensity with heat filter and neutral density filter was suspended above the tube. The light intensity was selected using a Weston Master III light meter, and five ml. of a suspension of fully developed eggs was transferred to the vessel in total darkness. The eggs were allowed to sediment and the tube was illuminated. At three minute intervals the surface layer of water (one inch) was removed using a pipette and the volume made up with fresh distilled water. The

miracidia in each sample were killed by the addition of a few drops of 5% formalin. In this way it was possible to localise the time after illumination at which eggs hatched.

The effect of intensity on the rate, and on the total percentage of eggs hatching, was also estimated. Stimulation of hatching by other factors, in the absence of light was also recorded. Exposure of eggs to daylight was used as a control.

(c) The Role of Osmotic Pressure.

i. Correlation of osmotic pressure and failure to hatch.

The osmotic pressure of the external medium of the egg was varied using a series of sodium chloride solutions (0.1 - 1.0%). Eggs were transferred to the solutions and allowed to hatch. After thirty minutes, miracidia and unhatched eggs were killed and the total number of free miracidia, and the number of eggs in which the hatching mechanism had malfunctioned, were counted. Controls consisting of eggs in distilled water, were used and each determination was repeated three times.

In conjunction with this experiment, the effect of saline was observed under the compound microscope.

ii. Expansion of the cushion.

The nature of the cushion expansion prior to hatching was investigated. Eggs were placed in different concentrations of saline and observed under the compound microscope. The length of time between expansion of cushion and rupture of the operculum was noted at each concentration. The

effect of saline on the final volume of the free cushion was also examined.

The cushions persisted for some time after hatching, and the numbers present were recorded at hourly intervals. Eggs were allowed to hatch in solid watch glasses containing distilled water and these were left for the requisite time. A few drops of dilute solutions of basic dyes such as Neutral red, etc. were added and the free expanded cushions were visualised. The number present was counted together with the total number of miracidia (= the number of cushions originally present).

iii. Emergence of the miracidium.

Rowan considered that the hypertonicity of the egg contents was responsible for expulsion of the miracidium. To test this hypothesis the effect of various salinities on the time taken for the miracidium to emerge (i.e. the time from rupture of the operculum to complete escape of the miracidium) were noted. The process was also observed under the compound microscope and the effect of the osmotic pressure of the external medium on the sacs, miracidium, etc., was noted.

(d) The Nature of the Viscous Cushion.

i. Histochemical tests.

Rowan, on uncertain histochemical evidence, concluded that the cushion was protein in nature. In Part II of this work the basophilic properties of the cushion were observed, together with its fibrillar structure. A characterisation of the cushion material was therefore attempted using histochemical tests. Expanded cushions prepared in the form of the dry or

gelatine-supported smears were used, (as described in Part II).

In order to establish with certainty that the large granules were in fact nuclei, tests were carried out for the presence of RNA and DNA. The Feulgen test (Feulgen and Rossenbeck, 1924) and the Kurnick (1955) modification of the methyl green-pyronin technique were both used. Controls of cushions extracted with perchloric acid, prior to staining, were included. The difference in intensity between controls and test material could then be attributed to the presence of nucleic acid.

In order to determine the distribution of protein, the Mercury-Bromophenol blue test (Pearce, 1960), the Millon reaction for tyrosine (Baker, 1956) and the Sakaguchi reaction for arginine (Baker, 1947) were carried out on the dry smears only, because of possible interference from gelatine.

The carbohydrate content of the cushions was assessed using the tests described in Part IV, namely the P.A.S. reaction (McManus, 1946) on fixed material, and the Alcian blue and Toluidine blue techniques for acid mucopolysaccharides, on fresh cushions.

The lipid content of fresh cushions was investigated using a saturated solution of Sudan III in 70% alcohol.

ii. The chemistry of the cushion.

Enzyme digestions: Rowan (1957) used a variety of enzymes in attempts to digest the cushion and found that pepsin and trypsin did so, but hyaluronidase and diastase had no effect. He did not however use an assay procedure, nor did he discover if any particular component was attacked. (The transparent

nature of the cushion makes it difficult to assess the extent of digestion. Rowan's findings were extended using other enzymes, and on assay procedure which included 'visualising' cushions by addition of basic dyes. The enzymes used were trypsin, papain, hyaluronidase, neuraminidase and glucuronidase (for details see Wilson, 1964).

Purification of cushion material: The major component of the cushion was identified as acid mucopolysaccharide and this was investigated further. A suitable means of fractionating cushions was devised, based on differential centrifugation. Samples of 10,000 fully developed eggs were hatched, the miracidia were killed, and the solution was spun at 2000 r.p.m. for ten minutes. The supernatant containing the expanded cushions was removed and freeze-dried. The freeze-dried extract was then stored at -15°C. until required.

Electrophoresis: Rienits (1953) used paper electrophoresis to separate acid mucopolysaccharides and the methods used here were a modification of this procedure. The cushion extract was digested with proteolytic enzymes prior to concentration by freeze-drying. The experimental methods were similar to those used to examine vitelline cell protein in Part IV. An 0.05 M phosphate buffer, pH 6.7, was used with 0.125% Toluidine blue as a location reagent. Experiments of one hour's duration were performed at room temperature and a potential gradient of 5 volts/cm. was applied in all cases. Standard chondroitin sulphate (1 mg./ml.) was used for comparison with the cushion mucopolysaccharides.

Chromatography: One-way descending chromatography of hydrolysates was carried out in order to determine the sugar components. The principal

solvent used was Isopropanol-Water (Smith, 1960). Hydrolysates of cushion fractions were carried out overnight in 6 N.HCl at 105°C. No. 1 Whatman chromatography paper was used throughout and standard sugars were applied for reference purposes. Runs were usually of 18 hours duration.

Reducing sugars were visualised using Aniline phthalate reagent, followed by heating at 105°C. for ten minutes. Hexosamines were also detected using the specific Elson-Morgan reaction (Elson and Morgan, 1933) adapted by Cramer (1955) for chromatography.

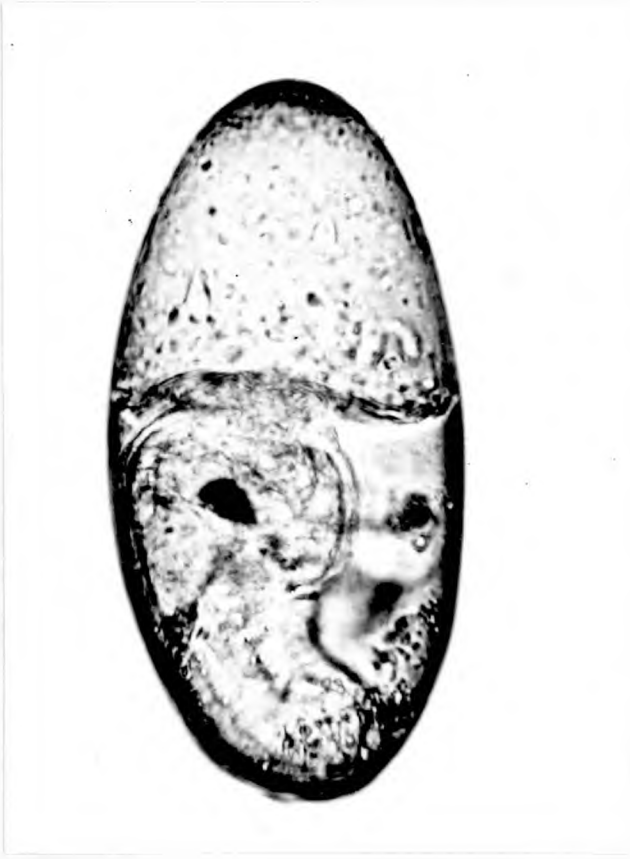
iii. Electron microscopy.

The fine structure of intact cushions, prior to hatching, was investigated using the electron microscope. The techniques used in preparation of material were identical with those described in Part III. It was found that phosphotunstic acid damaged the delicate fibrillar structure and staining with uranyl acetate was therefore employed.

Results.

(a) General Observations.

Stimulation of the miracidium results in expansion of the cushion after a delay of about five minutes (Plate 15). It is interesting to note that the expansion passes like a wave of solution through the fibrillar cushion matrix, and that in all cases observed the point at which expansion began was on the concave surface of the cushion, bordering the egg contents. It was never observed to expand from a point on the surface adjacent to the shell.



20 μ

Plate 15. The hatching mechanism; expansion of the viscous cushion.

After a pause of about 30 seconds the operculum ruptures violently - an indication that pressure is at least in part responsible for the process. The cushion material streams out of the egg (Plate 16) and takes up a characteristic hemispherical shape, about 120 μ in diameter. In the greatly expanded state the cushion appears amorphous apart from the very evident 'nuclei'. Rowan stated that the cushion did not form when eggs were hatched in distilled water, but simply flowed away. All his experiments were carried out under the compound microscope using slides with coverslips, and under these conditions the cushion is disrupted. When eggs were hatched in solid watch glasses, volume increases were much greater but no dispersal of the cushion complex occurred. It would seem, therefore, that the confining influence of the coverslip causes distortion and disruption of the cushion complex and any conclusions drawn from experiments performed under these conditions must be regarded with caution.

The miracidium emerges from the egg (Plate 17) and after a brief pause swims away. Its emergence is connected with the expansion of the sacs and after its exit these burst. (They may be rendered stable by allowing eggs to hatch in 0.7% saline (Plate 18)).

(b) Stimulation of the Miracidium.

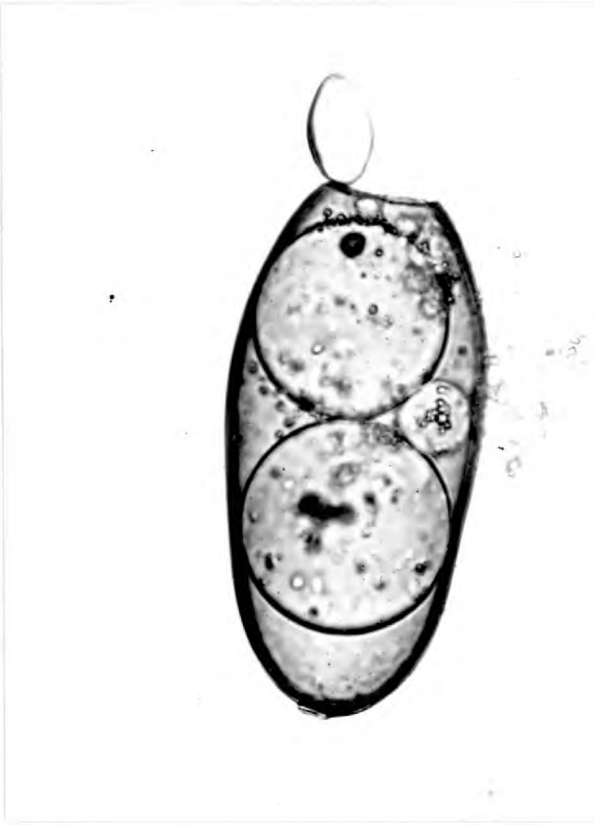
The numbers of miracidia hatching under different light conditions were counted at three minute intervals. The figures expressed as a percentage of total hatch are tabulated below. (Intensities (I) are given in units of the Weston Master III light meter).

20 μ

Plate 16. The hatching mechanism; rupture of the operculum and expulsion of the viscous cushion.

40 μ

Plate 17. The hatching mechanism; escape of the miracidium.



20 μ

Plate 18. The hatching mechanism; expansion of the 'sacs'.

Table XII.

Time \ I	6.5	13	100	800	Control
3 mins	0	0.4	0.5	0.02	0.89
6	0.9	3.3	3.4	5.9	1.78
9	1.6	25.7	23.9	21.6	16.0
12	12.9	24.9	26.8	22.7	20.5
15	43.1	20.2	16.0	15.8	17.2
18	11.2	12.2	12.8	10.5	16.3
21	3.2	6.4	6.8	9.5	11.3
24	6.4	4.1	4.0	5.4	7.1
27	4.8	1.9	4.3	5.1	2.3
30	14.5	0.8	1.6	3.3	2.0

The results of the control experiment are plotted in Fig. 18a to show the typical response. The optimum time for hatching is from 6 - 12 minutes at all intensities. The slow fall-off in numbers could well be a reflection on the technique used to remove the miracidium from the experimental vessel. The slight changes in hatching time at the intensity of 6.5 could also be the result of poor phototaxis rather than a real variation in hatching time.

The interesting feature which varied with light intensity was the percentage of eggs hatching. In a second experiment the following data were recorded: 6.5 units, 49.5% hatch; 13 units, 54% hatch; 100 units, 69.2%

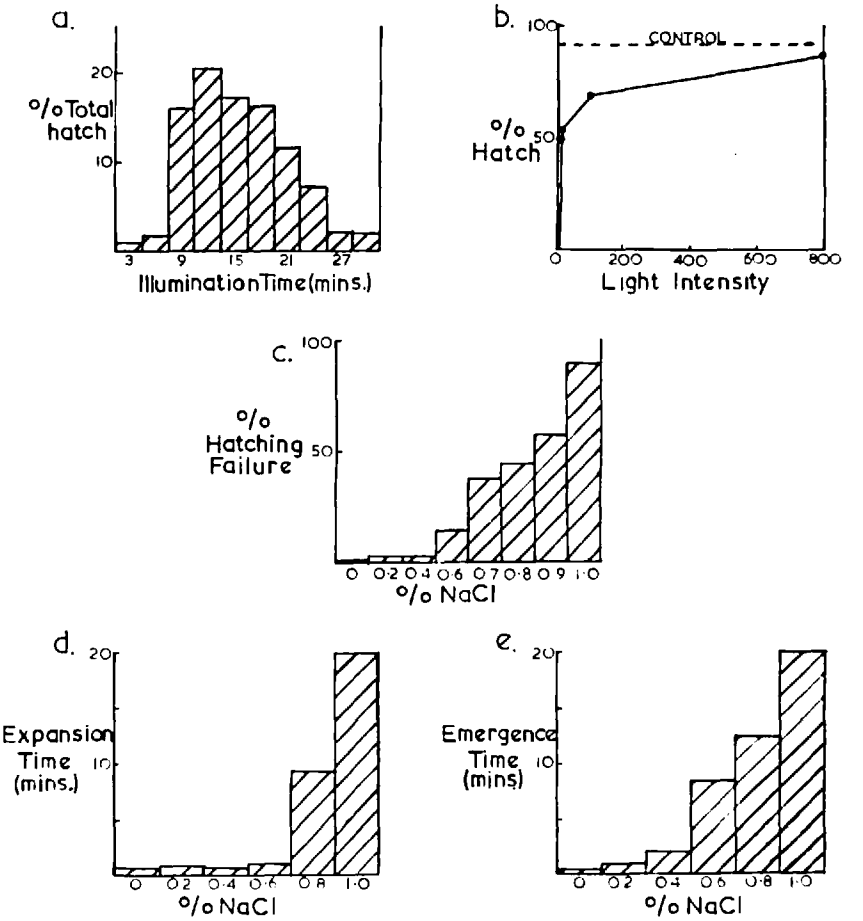


Fig.18 The Hatching Mechanism
- a. light and hatching time
- b. " " " percentage hatch
- c. osmotic pressure and hatching
- d. " " " " " cushion expansion
- e " " " " " emergence of miracidium

hatch; 800 units, 88.2% hatch; control, 96.4% hatch. The figures are plotted in Fig. 18b. The configuration of the graph is typical of what might be termed a trigger reaction. The great sensitivity of the miracidial eyespot is also evident, since intensities a little below 6.5 are at the threshold of human vision.

Friedl (1960, 1961) reported that exposure of eggs of Fascioloides magna to an atmosphere of nitrogen will induce hatching. Quite by chance it was found that the eggs of Fasciola could also be stimulated to hatch in the absence of light. Prolonged vibration of the culture vessels and also abrupt changes of temperature would serve as suitable stimuli though they were by no means as efficient as light. It would seem therefore that activation/hatching can be brought about by various stimuli which act as a trigger reaction.

(c) The Role of Osmotic Pressure.

i. The correlation of osmotic pressure and failure to hatch.

Eggs were immersed in different concentrations of saline and after 30 minutes the number of eggs in which the hatching mechanism had failed, was recorded. The results are tabulated below (Table XIII).

The results are illustrated by a histogram in Fig. 18c. It was apparent that almost all eggs failed to hatch in 1.0% saline and the reason for this was discovered when eggs were observed under the compound microscope. Below a concentration of 0.6% saline no effect could be detected. Above this concentration the cushion expanded to a greater extent often occupying two-

Table XIII.

Concentration of Saline (%)	Percentage Failure
Distilled water	1.5
0.2	3.3
0.4	2.9
0.6	15.2
0.7	38.0
0.8	45.6
0.9	57.9
1.0	89.6

thirds of the egg. The sacs were also flaccid and this probably accounts for the increased cushion expansion, no resistance being offered by the egg contents. The operculum does not rupture in 1% saline and this is not in accord with the presence of a hatching enzyme. The opercular seal is weakened, since the cap can be more easily dislodged, but it is never completely freed as one would expect if a hatching enzyme were present. In cases where the operculum is dislodged in 1% saline, the cushion remains within the egg. It is clear therefore that the hypertonicity of the egg contents must be in part responsible for rupture of the operculum and extrusion of the cushion.

ii. Expansion of the cushion.

Eggs were observed in different concentrations of saline and the

time between expansion of the cushion and rupture of the operculum was noted. Ten eggs were observed at each concentration and mean results are given below.

Table XIV.

Concentration (% Saline)	Time (seconds)
Distilled water	41.7
0.2	61.9
0.4	47.0
0.6	73.17
0.8	572.3
1.0	1200

If the operculum had not ruptured after 20 minutes, a figure of 1,200 seconds was recorded. The results are plotted in Fig. 18d.

It is clear that swelling of the cushion is not inhibited by increased saline concentrations, and as stated earlier, the actual volume occupied by the cushion is greater at high salinities. The osmotic pressure of the external medium does, however, have some effect on the final volume of the free cushion which is, in distilled water, 2 - 3 times that of cushions in 1.0% saline.

Nevertheless, even in 1.4% saline it was not possible to cause a complete reduction in volume to pre-hatch dimensions.

The cushion persists for some time after hatching and the percentage of the original total present was recorded at hourly intervals (Table XV).

Table XV.

Time after Hatching	Cushions (as % of miracidia)
1 hour	87.5
2 hours	84.0
3 "	79
4 "	70
5 "	76
6 "	57
7 "	57
8 "	53

The experiments were carried out in distilled water and the fact that 53% of cushions could be detected eight hours after hatching, completely refutes Rowan's observation that the cushion dissipates in distilled water.

iii. Emergence of the miracidium.

Eggs were hatched in various salines and the time from rupture of the operculum to escape of the miracidium was noted under the different conditions. The results are tabulated below (means of ten determinations) and plotted in Fig. 18e.

Table XVI.

Concentration	0%	0.2%	0.4%	0.6%	0.8%	1.0%
Time (seconds)	28.4	61.6	117.7	525	745	1200

From 0 - 0.4% saline the emergence time is little affected. At concentrations of 0.6% and above the sacs are flaccid and cannot participate by swelling. The miracidium is not therefore expelled immediately but must escape under its own exertions. The expansion of the sacs must therefore represent the chief agency in expulsion of the miracidium. Further proof for this is provided by miracidia which face the wrong direction and are expelled backwards. A similar mechanism of expulsion will probably be found to exist in a variety of Platyhelminthes such as the pseudophyllid tapeworms which have operculate eggs containing large ciliated larvae.

(d) The Nature of the Viscous Cushion.

i. Histochemical tests.

The large granules of the cushion were stained pink by the Feulgen reaction but the ground substance had no affinity for the Light Green counter-stain. This test was taken as confirmation that the larger granules were nuclei. Results of the Methyl green-Pyronin technique supported these findings. The nuclei were coloured green and the ground substance red. Extraction procedures removed the positive colouration of the nuclei but the ground substance was not affected. It must therefore consist of a baso-philic component other than RNA.

Both Millons and Sakaguchi reactions gave negative results. The less specific bromophenol blue test stained the nuclei bright blue (basic protamines and histones). The ground substance however gave only a very faint positive reaction even after prolonged staining, and this was localised

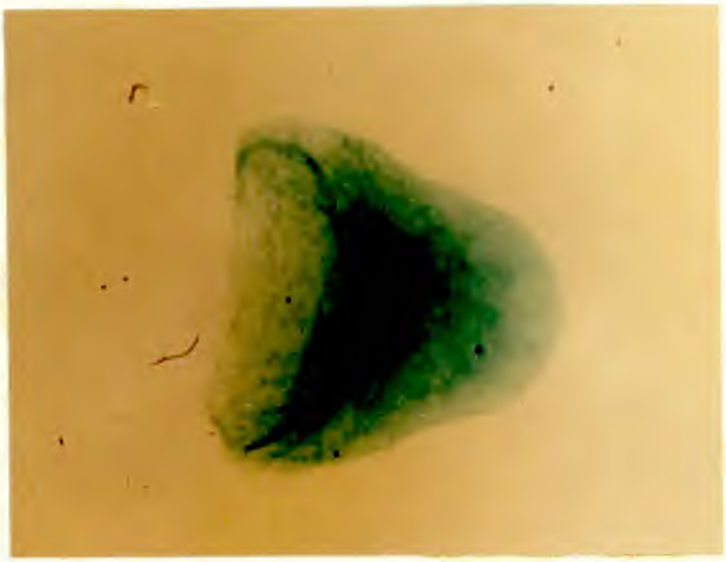
in areas less than 1 μ in diameter.

The PAS reaction gave negative results with the dry smears, but Toluidine blue and Alcian blue both stained the ground substance intensely (Plate 19). The Toluidine blue produced a strong γ metachromasia indicating acid mucopolysaccharide. Very dilute solutions of both dyes gave a positive reaction and increased concentrations resulted in a complete reversal of the swelling, which had occurred during hatching. This reversal may in some way be connected with the acidity of the ground substance. (Toluidine blue is thought to combine with the SO_3H and COOH groups of acid mucopolysaccharides).

On histochemical evidence it was therefore concluded that the cushion consisted principally of acid mucopolysaccharide with a minor protein component, and that nuclei are definitely present in the fibrillar matrix.

ii. The chemistry of the cushion.

Enzyme digestions: Addition of trypsin solution to water containing cushions caused them to fragment almost immediately. The action of trypsin on Toluidine blue stained cushions was observed under the microscope. The fibrils broke up into small fragments and finally dispersed, but the stain was not released by its action. It was concluded that the 'backbone' of the cushion consisted of protein strands, and the rapid fragmentation implied that they were an aggregation of a few molecules only. The mucopolysaccharide is probably attached to the protein by stable linkages (as is indicated by the duration of the cushion after release from the egg).



40 μ

Plate 19. The free viscous cushion, stained with Alcian Blue to show acid mucopolysaccharide.

Neither hyaluronidase nor neuraminidase had any noticeable effect on the cushion. This means that the mucopolysaccharide was neither hyaluronic acid nor chondroitin sulphate A or C and that it was not attached to the protein chain by a terminal neuraminic acid residue as is often the case. On the other hand β glucuronidase completely digested the cushions within one hour and this was taken to indicate the presence of a polyglucuronide or a compound with glucuronic acid as a constituent.

Electrophoresis and Chromatography: Electrophoresis of trypsin digests of cushions gave variable results. The sample migrated towards the anode but did not form discrete spots. In one hour the mean distance migrated was +0 to 1.4 cm. and this indicated a polydisperse product. Chondroitin sulphate migrated about 2 cm./hr. under the same conditions and this suggests that the cushion sample had either lower acidity or greater molecular weight.

Chromatography of cushion hydrolysates showed that two sugars were present. One was identified as a residual oligosaccharide and the other tentatively as 'hexosamine'. The R_L (Lactose) values were 0.2 and 1.9 respectively. Papers visualised by the Elson-Morgan method yielded a single pink spot, and R_H's (Hexosamine) of 1.1 and 1.2 were recorded. Hydrolysates of chondroitin sulphate had an R_H of 1.1. It was concluded that the unknown was an aminosugar, possibly glucosamine.

The amino-sugar content of undeveloped 9 day-embryonated, and fully developed eggs was determined colorimetrically by the Elson-Morgan reaction. Samples of 3000 eggs were hydrolysed and aliquots assayed. (There was considerable interference from amino acids which produce a yellow colour, as

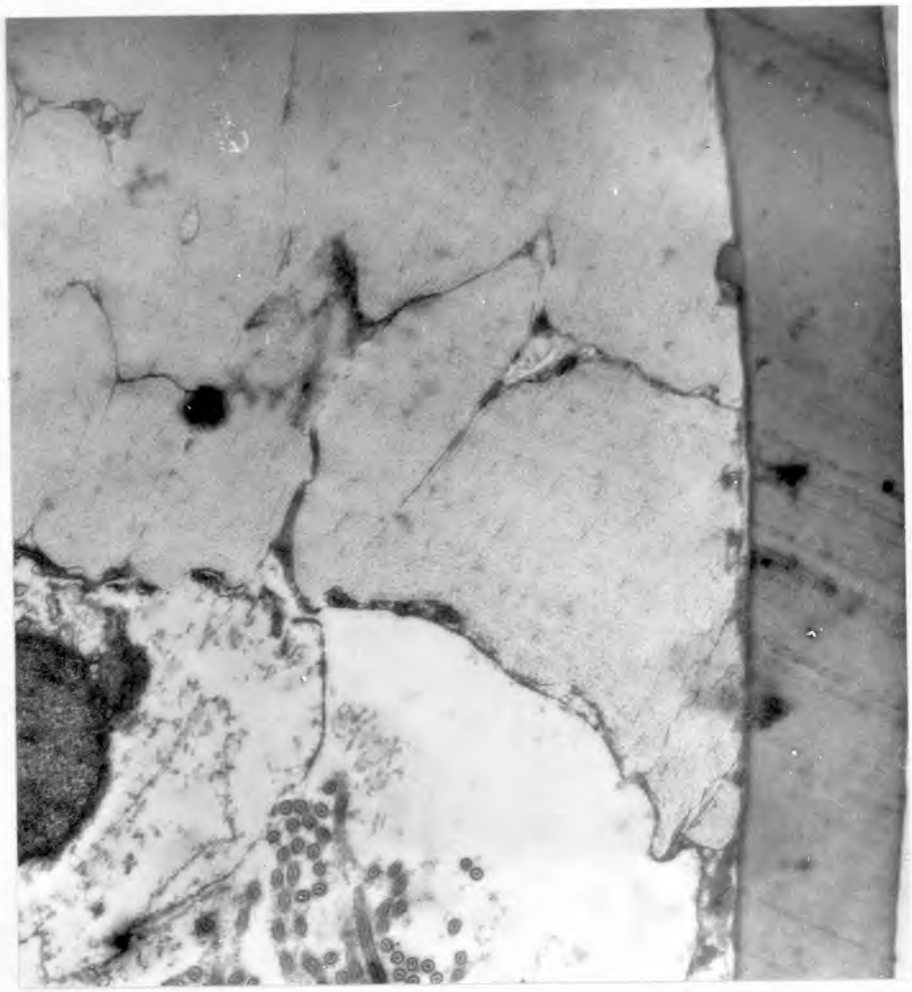
opposed to the pink of amino sugars). The undeveloped eggs were taken as tissue blanks and subtracted from other determinations. 9 day eggs contained 0.9 µg/1000 and fully developed eggs 1.5 µg/1000. This is equivalent to about 2% of the dry weight of egg contents.

iii. Electron microscopy.

The fibrillar nature of the cushion, the apparent solution of the fibrils at expansion, and the origins of the cushion within the vitelline membrane, have all been reported. The presence of membranes and nuclei associated with the cushion supported a cellular origin. The chemical components of the cushion have been investigated and it was considered that an examination of the fine structure of the cushion would be worthwhile.

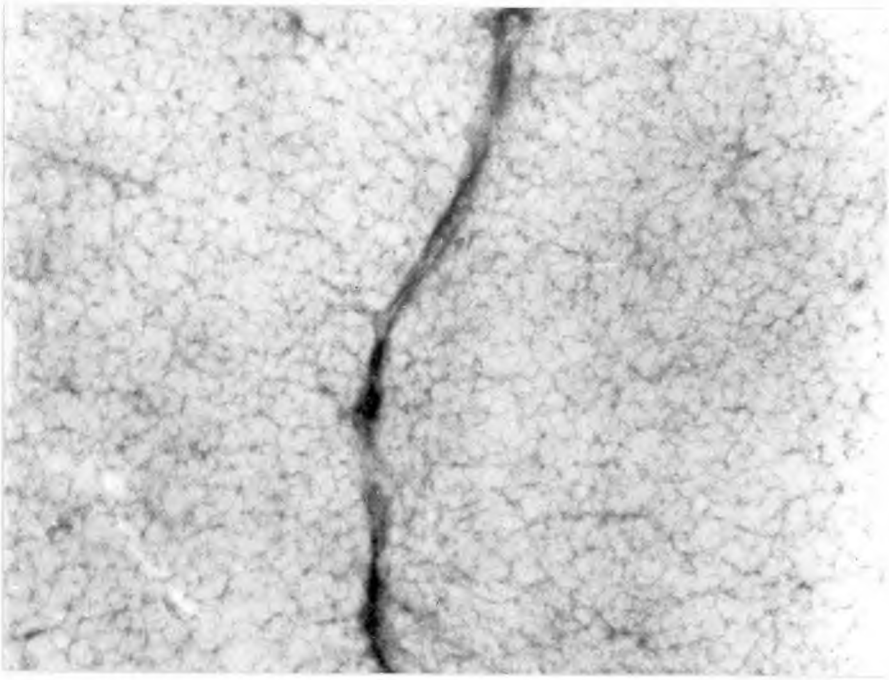
A general view of the cushion at low magnification is given in Plate 20. Coarse fibrils about 0.1 µ in diameter can be seen running through the matrix. Their appearance, when cushions are viewed under the light microscope, must be the result of interference phenomena as they are below the resolution of the light microscope. The ground substance appears to be slightly fibrous in nature and examination at a higher magnification reveals a reticulated structure (Plate 21) throughout the whole cushion. The average length of each basic unit is 400 Å and breadth 25 - 60 Å. These small fibrils also have a granular appearance but this could be an artifact produced by the embedding medium.

The cushion is delimited on all surfaces by a typical unit membrane (Plate 22). Traces of structures such as granules, membranes, etc. can also



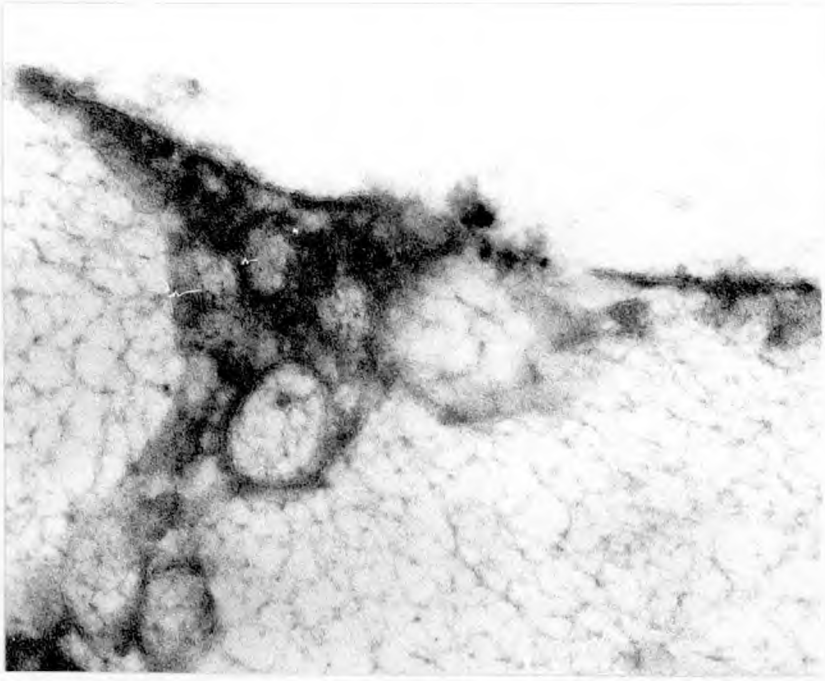
1 μ

Plate 20. Electron micrograph of the viscous cushion showing strands and inclusions.



200 mμ

Plate 21. High power electron micrograph to show the fine reticulum lying between the coarse fibrils.



80 mμ

Plate 22. Electron micrograph of inner, concave surface of viscous cushion, showing the limiting unit membrane.

be seen at various points in the matrix and probably represent the remnants of cell components.

Expansion of the cushion presumably occurs as a result of the elongation of the basic units. Uranyl acetate was chosen as a 'stain' for electron microscopy because it is a cation and would associate with polyanions such as acid mucopolysaccharides. The electron density of both coarse fibrils and fine reticulum must therefore be partly associated with the polyanionic complex.

Discussion.

The implications of the experimental results make it possible to put forward a new interpretation of the hatching mechanism. It is clear that the process is initiated by the receipt of an external stimulus. Light is the most efficient, but others such as vibration, temperature change, and according to Friedl, an atmosphere of nitrogen, will all serve the purpose. The effect of the stimulus is to activate the miracidium, presumably via the nervous system. This activation is most apparent in the epidermal cilia and flame cells. It was shown in Part V that the increase in flame cell activity was repressed by raising the light intensity and could not therefore be associated with an increase in osmotic work. Removal of the inhibition is followed, in some cases almost immediately, by expansion of the cushion and once this has happened nothing short of drastic measures will prevent hatching.

The viscous cushion is a highly organised and functional structure and it is hard to believe, with Rowan, that it plays only a passive role in hatching. Rather, its expansion would seem to be the focal point in the

hatching mechanism and its structure and properties therefore of great importance. It is not, as Rowan supposed, a homogeneous protein gel but in fact a fibrillar mucoprotein complex.

The volume of the expanded cushion within the egg varies directly with the osmotic pressure of the external medium so that cushion size was greatest at high salinities. It is clear from the plasmolysis caused by salines of 0.8% and above, that water will readily adjust across the 'vitelline membrane complex' to equalise internal and external osmotic pressures. The greater expansion of the cushion at higher salinities must therefore be attributed to lack of resistance in the shrunken egg contents. (The sacs progressively decrease in volume with increase in external osmotic pressure).

It is also clear that the volume of the free cushion is only partly related to the osmotic pressure of the medium. Thus, the volume of a free cushion in distilled water is about three times that of a cushion in 1% saline.

The properties of the cushion must be explained in terms of its chemical constitution. The major component is acid mucopolysaccharide attached to a minor protein fraction. The surface density of negative charges on the mucopolysaccharide molecule can be inferred from the histochemical results. The δ metachromasia produced with Toluidine blue indicates a surface density of 4 - 6 Å i.e. one acid group per sugar molecule.

Enzyme digestions confirmed the presence of a protein backbone responsible for the structural integrity of the cushion. The presence of a uronic acid component was also indicated.

It was not possible to prepare a homogeneous sample for electro-

phoresis but the product obtained seemed to be of fairly high molecular weight. A hexosamine component was also tentatively identified by chromatographic techniques and was determined colorimetrically to account for 1.5 µg/1000 eggs.

A consideration of these findings in terms of mucoprotein chemistry explains many of the cushion properties. It is thought that acid mucopolysaccharide molecules behave as random coils in the tissues and because of their electronegativity and coiling can retain 200 - 500 times their weight of water. The expansion in cushion volume was therefore interpreted as hydration of a mucoprotein complex.

There are two current theories which seek to explain the relationship of the mucopolysaccharide to the protein component. The first is that the protein forms a backbone chain along the length of which mucopolysaccharide molecules are attached. The second postulates that mucopolysaccharide and protein are arranged end to end. It is easy to visualise that hydration of a fibrillar system organised on either of these lines would result in a rapid elongation of the fibril.

Electron microscopy revealed that the cushion is ramified by large fibrils between which lies a fine reticulum. The basic units of this reticulum were 400 Å X 25 - 60 Å the greater part of which must be acid mucopolysaccharide. It is therefore postulated that hydration of the subunits results in elongation and all-round volume increase.

The electron microscope also confirmed that the cushion was formed between the vitelline membranes and showed that these were probably the

flattened surfaces of cells. The presence of nuclei was further evidence for the cellular origin. The cushion is thus completely surrounded by unit membranes which separate it from the egg contents.

It appears that prior to expansion the cushion is in a dehydrated or semihydrated state, and hence, occupies minimal volume. The actual expansion is interpreted as hydration and consequent volume increase of the fibrillar network. This is in complete accord with the observation that at expansion, "a wave of solution" passes through the matrix.

The process is evidently not completely physical in nature and a reversal of expansion, whilst not brought about by osmotic pressure alone, can be caused by dilute solutions of basic dyes such as Toluidine blue. It is possible therefore that removal of a natural molecule of similar function to Toluidine blue allows the cushion to hydrate.

The actual point at which expansion begins was found to be the concave surface nearest the egg contents and hence the miracidium. It is postulated, therefore, that the miracidium is directly responsible for initiating the process by actively damaging the surrounding membrane, allowing communication between either egg contents or external medium, and cushion matrix. Plasmolysis showed the egg contents to have an osmotic pressure approximately equivalent to 0.7% saline and the cushion will still expand when the external medium if of this concentration. There is therefore no need to postulate that an influx of external medium is necessary for cushion hydration since the fluid of the egg contents will probably be sufficient, under natural conditions.

The expansion of the cushion must produce considerable pressure within the egg since the miracidium and sacs normally offer resistance. The time lag prior to rupture of the operculum can be interpreted as a build-up of this pressure and evidence is forthcoming in the fact that the duration of cushion expansion increases with external osmotic pressure. Thus, in distilled water, the mean time is 42 seconds, in 0.6% saline 73 seconds, and in 1.0% saline the operculum is weakened but does not rupture.

Rowan's evidence cannot be ignored and the possibility of a hatching enzyme dissolving the opercular bond must be considered. As evidence for such an enzyme, Rowan stated that compression of eggs by coverslips resulted, not in rupture of the operculum but in a simple splitting of the egg shell. If however eggs were punctured and digested in a solution of proteolytic enzymes, the operculum was loosened and could be more easily dislodged. He also stated that in punctured eggs exposed to the "metabolites from hatching eggs, the opercula ..... loosened during subsequent compression beneath the coverslip".

In all observations on fluke eggs made here, it was noted that compression almost always resulted in a partial rupture of the opercular bond. It is emphasised that the production of a pressure within the egg and directly beneath the operculum would have a different effect to compression of an egg from without. The loosening of the operculum in trypsin solution was not as marked as Rowan suggests but could possibly indicate that the opercular discontinuity was partly composed of protein. No histochemical evidence was found for this. The fact that metabolites of hatching eggs were said to

loosen the opercular bond is harder to explain. It is possible that in the confined space of the capillary tubes used, the free miracidia had released the substances with which they are thought to cytolyse the snail epithelium. Certainly, miracidia have been observed to secrete the contents of the primitive gut when trapped in the meshes of lens tissue.

Another reinterpretation of a part of Rowan's data can be advanced. He discovered that expansion of the cushion could be brought about by external factors such as heat, toxic chemicals, etc., all of which would damage the surrounding membrane. The cushion expands but the operculum does not rupture. This was interpreted by Rowan as non-release of the hatching enzyme. It is equally possible to postulate that the external factors had damaged the vitelline membrane complex allowing egg contents to diffuse out. Thus as the cushion expands, this process is accelerated and there is no pressure build-up capable of destroying the opercular bond.

Two positive criticisms of the hatching enzyme theory were advanced in the introduction to this section. They were, the accessibility of the opercular bond, and the fact in an egg hatched in 1% saline, the operculum does not rupture. To reach the operculum, an enzyme released by the miracidium would have to pass through at least two membranes and a layer of perivitelline material, and possibly in addition the matrix of the viscous cushion. If the postulated enzyme were capable of reaching the opercular bond in sufficient quantity to be of any value then it would almost certainly destroy the viscous cushion in the process. As to the second criticism, it is unlikely that 1% saline would inhibit an enzyme and it is therefore concluded that pressure in

part at least, is responsible for rupture of the operculum. The hypothesis, that a hatching enzyme dissolves the opercular bond, therefore seems untenable.

The hypertonicity of the egg contents is responsible for the expulsion of the viscous cushion and miracidium. The main agents are the 'sacs', usually 2 - 5 in number, which are the last remnants of the vitelline cells.

The final interpretation of the hatching mechanism will be possible only when more is known about the changes in permeability of the vitelline membrane - perivitelline material complex during hatching.

A solution of this problem may lie in the application of density-gradient techniques, or the use of Cartesian-diver balances to determine changes in density during hatching.

In conclusion the above interpretation of the hatching mechanism is summarised:

Light stimulates the miracidium and when activation is complete an 'operative factor' is released which is responsible for altering the permeability of the membrane on the internal concave surface of the viscous cushion. Prior to expansion the cushion (a fibrillar mucoprotein complex) is in a dehydrated or semihydrated state. The change in permeability of the enclosing membrane allows the fluid egg contents to permeate into the cushion. The fibrillar matrix becomes hydrated (either by removal of a natural inhibitor, or as a direct result of influx of fluids), and an increase in volume occurs. The miracidium and sacs are compressed and internal pressures set up. These increase until the operculum finally ruptures under the strain and flies back violently. The cushion and miracidium are then expelled by the hypertonicity of the egg contents.

PART VII. THE BEHAVIOUR OF THE MIRACIDIUM.

Introduction.

Several investigations have been carried out into the factors affecting the orientation of the miracidium. Wright (1959) has summarised the data and suggested that the miracidial behaviour is divided into three phases. The miracidia hatch and rise to the surface (positive phototaxis). This is followed by a period of random movement which brings the miracidium into the orbit of the snail. The miracidium then locates the snail by a chemotactic process.

The first part of the hypothesis, namely the positive phototaxis of the miracidium has been demonstrated many times. Yasuraoka (1953) showed that up to 16 hours after hatching 88% of the miracidia of Fasciola hepatica remained in the upper stratum. After 16 hours the mortality increased and miracidia sank to the lower layers. After 24 hours all were dead. He also determined that geotaxis was a contributory factor in the rise to the surface, as miracidia hatched in darkness also congregated in the upper layers. The effect did not however persist for long and after seven hours the miracidia were evenly distributed throughout the vessel.

The miracidia of Fasciola possess well developed eyespots but attraction to light is also shown by the miracidium of Schistosoma which have no apparent light sensitive organs. Standen (1951) observed the effect of light on hatching, in S. mansoni and proved that the miracidia were definitely positively photactic. Etges and Decker (1963) concluded that light and gravity were the two most powerful stimuli affecting the miracidium of

S. mansoni. There is quite possibly a light sensitive organ in these forms with no obvious eyespot. The pigment cup of the miracidium of Fasciola serves to make the organelle more efficiently directional, and as a consequence renders the eyespot visible.

The latter part of Wright's hypothesis, namely the random movement and chemical attraction to the snail is more speculative. The movement of the miracidium under laboratory conditions is quite probably random, but under natural conditions such factors as water currents (Neuhaus, 1941; Yasuraoka, 1953), and temperature (Takahashi, 1961) must affect its orientation.

Reports concerning a chemotactic response are by no means unanimous in their conclusions. Smyth (1962) states that "There is no evidence that a miracidium finds its host by chemotaxis". Campbell and Todd (1955a) could find no response by the miracidium of Fascioloides magna to the snails Fossaria and Stagnicola. Mattes (1936, 1949) concluded that miracidia distinguished objects by their texture alone, and would not attempt to penetrate hard materials. On the other hand various snails, turbellaria, annelids, insect larvae, hydrozoa, and tadpoles were all attacked with equal vigour, penetration being effected in snails only.

Other workers have conclusively demonstrated chemotactic responses by the miracidium. Neuhaus (1941, 1953) stated that the miracidium showed positive rheotaxis to the ciliary currents produced by the snail, and chemotaxis was evident at a distance of 12 - 16 cm. from the snail host. Similar positive chemotaxis was demonstrated by Kloetzal (1958) in the miracidium of S. mansoni for Australorbis glabratus. Reports on the specificity of the

response are also variable. Neuhaus concluded that related species of snail were equally attractive but Etges and Decker state that for S. mansoni to Australorbis it was absolutely specific.

The penetration of the larva of F. hepatica and F. gigantica into the snail host was described by Dawes (1959, 1960a, b, c). The miracidium attaches to the snail by the suctorial action of its anterior papilla. The snail's epithelium is then cytolysed and about 30 minutes after attachment the larva enters the snail. In the process the ciliated epidermal cells are lost so that it is in fact the sporocyst which enters. Dawes was of the opinion that the granular contents of the primitive gut were the "precursors of digestive secretion". The actual stimuli which bring about metamorphosis are unknown but Campbell and Todd (1955b) observed the process in vitro by the miracidium of Fascioloides magna.

It was hoped to study the factors affecting the miracidium and its mode of penetration into the snail, in detail. However, sufficient time was not available to complete the work and only the preliminary observations were made.

Observations and Conclusions.

The presence of a phototactic response can very easily be tested by varying the direction of a light source. The miracidia will swim towards the source, at the same time oscillating slightly on their short axes. Taxis to a light source demands two or more light sensitive organs so that intensities can be compared. The eyespots presumably serve this function and the rolling

motion of the miracidium must result from alternating stimulation of epidermal cilia on opposite sides. The mechanism is thus self-correcting. The phototaxis is stronger than geotaxis and illumination of the vessel from below causes the miracidia to congregate at the bottom.

The effect of the system is to bring the miracidium to the surface of the puddle or pond, after hatching. Here light intensity is uniform and unless other factors interfere the miracidium will move randomly until it collides with an object, after which the course is changed. Neuhaus and Yasuraoka alone seem to have realised the importance of water currents. Even the slightest movement of water will cause the miracidia to orientate into the current, and it appears that the reaction will override all other taxes. Its effect on the search for the snail host has not been determined but must be profound. Ponds are seldom without some ripple, or surface movement of water and the miracidium would therefore move "upstream".

The behaviour of the snail host is equally important but has not attracted attention. Experiments aimed at discovering how the snail is located should not neglect the behaviour of the snail. Pulmonates are generally found on the surface, and submerged near the edges of ponds. The net result of the various factors affecting the miracidium is probably to bring it to these areas where contact with the snail can be made.

Observations on the chemotactic response indicated that there was a definite identification of snails. Tubellaria, etc., were not of interest to the miracidium but snails or even snail mucus would immediately cause excitement. The effect was not specific and the mucus of both limnaeids and

planorbids would elicit the response. It did not appear to operate from an appreciable distance and could not therefore be called chemotaxis.

Penetration occurred in several snail species and it was therefore concluded that specificity was host imposed. This could possibly take the form of an immune reaction of some kind. Some indication of the mechanisms underlying penetration was obtained from the fact that solutions of Maeyer's albumen, smeared on slides, would induce the miracidium to attempt penetration. The 'identification' must therefore be due to specific chemicals present in the snail mucus.

The secretion of the granular contents of the primitive gut has been described previously. Their basophilic nature and P.A.S.-positive staining reaction indicate mucoprotein components. Their similarity to vertebrate lysosomes has already been suggested.

Attempts were made, using the method of Charney and Tomarelli (1947), to detect proteolytic activity in homogenates of the miracidium. No positive reaction was obtained but this does not rule out the possibility as the technique was probably not sufficiently sensitive.

The epidermal cells are definitely released prior to penetration and can be observed 'swimming' around rapidly, until their energy sources are exhausted. Electron micrographs of the miracidia revealed that these cells were attached to the body at a few places only, large spaces lying between the points of contact. Their release must involve some form of intracellular cytolysis, triggered by a stimulus directly or indirectly due to the presence of the snail host.

It is clear that much remains to be discovered of the biology of the miracidium. It is intended to continue this work using modern techniques in an attempt to elucidate the underlying mechanisms which bring about successful penetration into the snail.

CONCLUSIONS

The 'free-living' stages of the Digenea are found in marine, fresh water, and terrestrial situations. It is remarkable, therefore, that they constitute such an apparently uniform assemblage from the point of view of morphology, physiology and life cycles. This implies a great functional plasticity of existing structures. The apparent variation in the function, but not the morphology, of the flame cell system is a case in point, and only a study of the comparative physiology of such organelles can shed light on the problem.

The relative difficulty in obtaining material for experimental purposes, has severely hampered research into the physiology and biochemistry of digenes. The obvious solution is the development of *in vitro* culture methods similar to those applied to bacteria and protozoa. The attempts made have all fallen short of this goal, probably due to insufficient knowledge of the fundamental biology, and so the circle cannot be broken. The experimenter is therefore forced to make the best use of available material. This must entail maintenance of at least some stages in the laboratory.

The first part of this work described the possibilities and problems involved in such a procedure. Most work on life cycles has followed an empirical approach. Whilst it has proved relatively easy to 'complete' a great many life cycles, the problem of maintaining the hosts and parasites in sufficient numbers for experimental work, has not been satisfactorily solved. The limitations on this, imposed by factors such as snail reprod-

uction, were noted.

Several benefits are conferred by the use of Fasciola as an experimental animal. There is a considerable body of literature on many aspects of the subject, and flukes can readily be obtained in large numbers from slaughter houses. The eggs can easily be recovered from flukes or bile in an uncontaminated condition. (This is not the case in intestinal flukes). Also, the intermediate host is maintained in several laboratories and can be fairly readily infected.

The role of an egg is the protection and nourishment of hereditary material during the period in which this is forming a new organism capable of existing by its own devices. An egg is therefore provided with food reserves and is usually surrounded by a resistant covering, the shell, which is responsible for the maintenance of integrity. Variations in this basic pattern have evolved many times allowing new situations to be exploited. The natural environment of the liver fluke egg is fresh water, and this entails at least certain modifications connected with the hyperosmotic condition of the egg contents. The unique type of egg found in many Platyhelminthes, consisting of an ovum plus several vitelline cells, may also have some functional advantage, but this is not at present obvious.

The liver fluke egg is surrounded by a shell some 1 - 2 μ in thickness. This was shown to consist solely of protein, 'tanned' to form a loose, but resistant, reticulum of fibrils. The shell material originates in the vitelline cells which contribute about 20% of their contents in its formation. It was found to be quite permeable to nearly all small molecules and its

primary function must be that of physical protection. It is interesting to note that although most bacteria and fungi are excluded, at least one, Catenaria anguillulae, is adapted to the environment provided by the fluke egg. The shell also serves as an exoskeleton, and weakening by hypochlorite results in a loss of egg configuration. A possible secondary function may result from the brown colouration. The shell 'pigment' absorbs strongly in the blue - U.V. region, so that it could serve to protect the egg contents from the harmful effects of ultra-violet radiation (particularly important in an embryo which contains a high concentration of hereditary material).

Directly beneath the shell of the undeveloped egg lies a layer of perivitelline material. This appears to have hydrophilic properties, and is probably analogous to the perivitelline material of other freshwater eggs. In forms such as the trout and frog this layer represents a barrier to outward diffusion. Kao thought that, as a result of its hydrophilic properties, a pressure was exerted against a limiting membrane, which resulted in a diminution of pore size and consequent reduction in permeability. The perivitelline material of the fluke egg appears to have hydrophilic properties and its function as a barrier against diffusion was suggested. Experiments with labelled phosphate supported this theory and also indicated that the shell, because of its porous nature, may retain fluids within the matrix.

The vitelline cells are produced in vitellaria, said by Hyman to be transformed ovaries, and this origin may account for their atypical chemistry. Most eggs are endolecithal and though yolk cells, whose principal role is nutrition, may result from cleavage, free, accessory, yolk cells are not

usually found. Each vitelline cell in the Fasciola egg contains a nucleus, possibly mitochondria, and a highly organised food reserve. The major constituents are protein (35%), carbohydrate (33%), and lipid (12%). About half the protein is in the form of a soluble homogeneous fraction, and most of the carbohydrate is present as glycogen. The high carbohydrate and low lipid contents are unusual.

A small undivided ovum is present in eggs removed from flukes, and this lies at or near the opercular pole. Its possible role in the formation of the opercular ring was suggested. It could secrete a substance in some way responsible for prevention of shell tanning at that point.

Oxygen, and a temperature above 10°C. are necessary before development is initiated. It is presumably the low oxygen tension of the intestine and faeces which delay development until eggs come into contact with fresh water. Morphologically, development can be divided into two distinct phases: firstly, a period of cell division, and secondly a period of cell differentiation resulting in the fully formed miracidium. The two phases are also reflected in the growth pattern of the embryo. The first phase is marked by an all-round volume increase, the second by a period of rapid elongation of the embryo.

At about the middle of development cells are said to break away from the embryo and to move to the periphery where they differentiate to form the vitelline membrane. This 'membrane' can be observed in fully developed eggs, lying beneath the shell, and is in fact two closely opposed membranes, thought to be the flattened surfaces of the peripheral cells. The

appearance of these membranes during development must have some effect on egg permeability. It could be that their presence is necessary to supplement the supposed hydrodynamic perivitelline barrier. It was discovered that the viscous cushion, which is formed beneath the operculum during the latter part of development, is laid down within the 'vitelline membrane'. This may represent a more definite function of the peripheral cells, and the presence of nuclei within the cushion reinforces the evidence for cellular origins.

During development approximately 40% of the egg contents are combusted, a high figure compared to other eggs, and the remaining 60% also represents some extraembryonic material such as the viscous cushion. The main sources of combusted material were carbohydrate and protein, in a ratio of 3 : 2 respectively. The process of embryonation is obligatorily aerobic. A study of oxygen consumption and calculation of the respiratory quotient revealed something of the underlying metabolic patterns. Activation of the ovum is attended by a slight increase in oxygen consumption; which then remains fairly constant during the phase of cell division. The later period of cell differentiation is accompanied by an increase in oxygen consumption and there is a final decline as development is completed. At the beginning of development the Respiratory Quotient decreased to a value of 0.48. This was correlated, both histochemically and by direct chemical analysis, with a synthesis of lipid. The mean RQ for the cell division phase was 0.81 (range 0.66 - 0.94), indicating a mixed substrate with carbohydrate predominating. The mean RQ for the cell differentiation phase was 0.68 (range 0.62 - 0.75),

indicating a lipid substrate. This latter was confirmed by histochemical means. The significance of the synthesis of lipid at the beginning of development, the correlation of cell division with a predominantly carbohydrate substrate, and the correlation of cell differentiation with a lipid substrate, are not understood.

The fully developed egg consists of the shell, perivitelline material, and double vitelline membrane, surrounding the miracidium, viscous cushion, and sacs. (These last are the remains of vitelline cells). The hatching mechanism was investigated in detail and the hypothesis put forward by Rowan is seriously questioned. The viscous cushion is a fibrillar mucoprotein complex in a dehydrated or semihydrated state, and admission of water, or the fluid egg contents to the matrix brings about hydration and volume increase. The agent responsible for damage to the containing membrane of the viscous cushion is thought to be released by the miracidium, since swelling of the cushion always commences in the region nearest the anterior end of the miracidium. It is suggested that the sudden expansion of the cushion creates pressure sufficient to rupture the operculum. The presence of a hatching enzyme digesting the opercular bond seems improbable, in view of the obstacles which must be circumvented and the damage likely to be caused. Pressure definitely plays some role in hatching since the operculum is not released when fully developed eggs are placed in 1% saline. The expulsion of the cushion and miracidium from the egg are brought about by the hypertonicity of the egg contents.

The hatching mechanism in both cestode and trematode eggs has been

subject to a great deal of speculation. It seems unlikely that the mechanism in the Fasciola egg is unique and a system based on the hydration of a mucoprotein complex probably exists in other parasitic platyhelminths which hatch free living larvae (e.g. Pseudophyllid tapeworms).

An investigation of the function of the flame cell system produced some confirmatory evidence for the hatching theory. The increase in flame cell activity at hatching, previously thought to be correlated with increased osmotic work, was shown to be inhibited by high light intensities. The activity of flame cells in the free miracidium could not be correlated with external osmotic pressure. The movement of the flame was shown to produce a hydrostatic pressure within the tubules, and the cell itself is probably the site of a primary filtration. Selective reabsorption is thought to occur in the tubule region. Control of osmoregulation could result from a constriction in the tubule at some point. The terminal atrium was never observed to pulsate in the manner of a bladder.

Various structures, not previously described, were observed in the miracidium. The terminal portion of the excretory duct was shown to be surrounded by a granular region and the presence of two large basophilic cells, behind the excretory pores, was noted.

The ultrastructure of the miracidium was not investigated but chance electron micrographs revealed the presence of pits, papillae, etc., (not described in the text) which could have a sensory function. A study of the miracidial behaviour was also confined to observations but the presence of photo-, geo-, and rheotactic responses, and a chemical 'sense' were all

recorded. It seems probable that the activity of the miracidium brings it into the environment of the snail where identification and penetration ensue. The specificity of the intermediate host seems to be host-imposed rather than the result of attraction to a particular snail.

An interpretation of miracidial behaviour must eventually depend on a knowledge of its sensory structures, etc., and also on a detailed knowledge of snail behaviour and physiology. It is hoped in further work to investigate these aspects, particularly the relationship of the miracidium/sporocyst to the snail host.

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APPENDIX I.

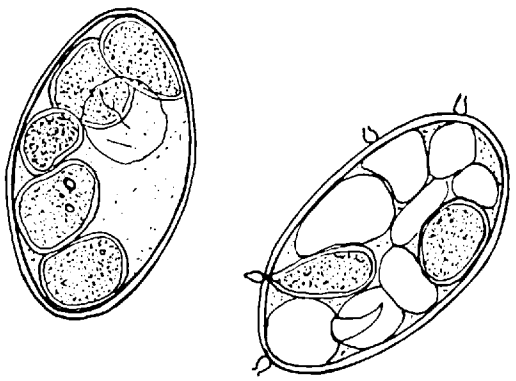
Parasites of Fluke Eggs.

As mentioned in Parts II and IV many of the cultured eggs failed to develop (up to 40%) and prolonged culture showed that some were infected with parasites (Fig. 19a, b).

The form illustrated in Fig. 19a occurred most frequently and was identified as Catenaria anguillulae, a slime fungus (Chytridales). This fungus was first described from the liver fluke egg by Butler and Buckley (1927), though it had been described earlier from various nematodes (see Dollfuss, 1946). The infection is spread by minute flagellate spores which adhere to the surface of the shell, penetrate and develop by utilising the egg contents. The hyphae consist of globular swellings connected by short narrow sections. From each globule a small hypha penetrates the shell and forms a cup-like body on the outer surface. The spores are shed via this aperture and further eggs are infected. The most interesting feature of the parasite is its apparent ability to dissolve the shell, in entering and leaving the egg. This must be effected either by the secretion of a powerful oxidising agent, or of an unusual enzyme capable of reversing the shell-protein tanning mechanism.

The second parasite (Fig. 19b) does not appear to have been described before. It also seems to be a species of fungus and dispersal is probably effected by the globular bodies. These are frequently found adhering to the surface of uninfected eggs.

a.



b.

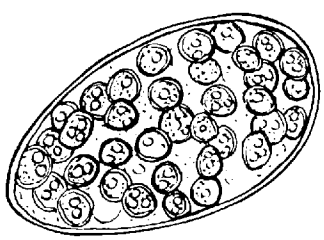


Fig. 19 Parasites of the Fluke Egg

The occurrence of these parasites presented a problem, as their growth processes could obviously not be separated from those of the developing eggs.

Several antibiotics were used without success, in attempts to eradicate the fungi. Up to this time, dechlorinated tap water and pond water had been used as a culture medium, together with unsterilised flasks. Substitution of distilled water and sterilisation of glassware proved to be effective measures, in completely eradicating the parasites.

The wide host range of the fungi and apparent abundance in natural waters, suggests that they may play a minor role in controlling parasitic helminth populations.

APPENDIX II.

Respiration of eggs, sample result.

Respiration of undeveloped eggs, i.e. Day 0. Respirometer Readings.

Tube \ Time		0 mins	15	30	45	60	90	120
TB	1	166.54	166.34	166.31	166.28	166.29	166.29	166.28
	2	169.58	168.89	169.31	169.48	169.80	170.27	170.23
a KOH	1	160.20	159.95	159.95	159.94	159.93	159.93	159.92
	2	172.55	169.99	170.33	169.37	168.60	166.59	164.35
b KOH	1	164.47	164.17	164.22	164.18	164.17	164.18	164.17
	2	118.03	116.75	116.00	115.0	114.16	112.18	109.81
c KOH	1	162.73	162.57	162.54	162.56	162.50	162.50	162.49
	2	131.67	130.34	129.74	128.91	128.22	126.26	123.92
TB	1	183.57	183.35	183.32	183.33	183.32	183.32	183.30
	2	152.50	151.90	152.13	152.18	152.36	152.37	152.28
TB	1	161.25	161.12	161.13	161.10	161.09	161.05	161.07
	2	153.40	153.19	153.68	154.0	154.25	154.58	154.92
a NO KOH	1	159.19	158.93	158.86	158.89	158.88	158.87	158.86
	2	155.21	154.50	154.46	154.20	154.13	153.60	152.98
b NO KOH	1	158.49	158.28	158.28	158.26	158.28	158.28	158.28
	2	152.03	151.22	151.32	151.17	151.03	150.48	149.81
c NO KOH	1	163.48	163.28	163.26	163.24	163.24	163.22	163.20
	2	132.04	131.63	131.73	131.70	131.65	131.35	130.97
TB	1	178.34	178.13	178.12	178.12	178.10	178.07	178.07
	2	140.00	139.85	140.26	140.66	140.90	141.28	141.71

(1 = Ref. mark, 2 = Indicator fluid). (Time in minutes, and measurements in mm. TB = Thermobarometer).
Tubes with KOH measure Oxygen uptake.
Tubes without KOH measure Oxygen taken up minus Carbon dioxide given off.

Actual Reading (i.e. subtraction of Line 2 from 1).

Tube \ Time	0	15	30	45	60	90	120
TB	3.04	2.55	3.00	3.20	3.51	3.98	3.95
KOH	12.35	10.04	10.38	9.43	8.67	6.66	4.43
KOH	46.44	47.27	48.22	49.18	50.01	52.00	54.36
KOH	31.06	32.23	32.80	33.65	34.28	36.24	38.57
TB	31.07	31.54	31.59	31.15	30.96	30.95	31.02
TB	7.85	7.93	7.45	7.10	6.84	6.47	6.15
No KOH	3.98	4.43	4.40	4.69	4.75	5.27	5.88
No KOH	6.46	7.06	6.96	7.09	7.25	7.80	8.47
No KOH	31.14	31.65	31.53	31.54	31.59	31.87	32.23
TB	38.34	38.28	37.86	37.46	37.20	36.79	36.36

Accumulated changes in Readings with Time (i.e. subtraction of Time 0 reading from other readings).

Tube \ Time	15	30	45	60	90	120
TB	-0.49	-0.04	+0.16	+0.47	+0.94	+0.91
KOH	-2.31	-1.97	-2.92	-3.68	-5.69	-7.92
KOH	-0.83	-1.78	-2.74	-3.57	-5.56	-7.92
KOH	-1.17	-1.74	-2.59	-3.22	-5.18	-7.51
TB	-0.47	-0.12	-0.09	+0.11	+0.12	+0.05
TB	-0.08	+0.40	+0.75	+1.1	+1.38	+1.70
No KOH	-0.45	-0.42	-0.71	-0.77	-1.29	-1.90
No KOH	-0.60	-0.50	-0.63	-0.79	-1.34	-2.01
No KOH	-0.51	-0.39	-0.39	-0.45	-0.73	-1.09
TB	+0.06	+0.48	+0.88	+1.14	+1.55	+1.98

Corrected Readings (i.e. experimental readings minus mean thermobarometer readings).

Tube \ Time	15	30	45	60	90	120	No. of eggs
1 KOH	2.83	1.81	2.96	3.97	6.22	8.40	1155
2 KOH	0.35	1.62	2.78	3.86	6.09	8.40	1190
3 KOH	0.69	1.58	2.63	3.51	5.71	7.99	1530
4 No KOH	0.44	0.86	1.52	1.89	2.76	3.74	1770
5 No KOH	0.59	0.94	1.44	1.91	2.81	3.85	1505
6 No KOH	0.50	0.83	1.21	1.57	2.20	2.93	1235

Mm moved/1000 eggs/1 hour.

1	3.64	
2	3.53	1 mm = 0.03 µl ∴
3	2.61	
Mean	3.26	× 0.03 = 0.0978 µl = O_2 taken up.
4	1.06	
5	1.26	
6	1.19	
Mean	1.18	× 0.03 = 0.0354 µl = $O_2 - CO_2$ given off.

$$\text{The Respiratory Quotient} = \frac{CO_2 \text{ given off}}{O_2 \text{ taken up}}$$

$$= \frac{(0.0978 - 0.0354) \times 1.1}{0.0978}$$

(1.1 is a factor allowing for CO_2 in solution).

$$\text{R.Q.} = 0.7$$