A STUDY OF THE ENVIRONMENT OF LARVAL *FASCIOLA HEPATICA* L.,

WITH A VIEW TO DEVELOPING A DEFINED MEDIUM FOR "IN VITRO" CULTURE

by

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ABSTRACT

An attempt is made to define the environment of the larval stages of Fasciola hepatica, parasites in the snail Lymnaea truncatula. An analysis is made of the host snail's haemolymph, including determination of all the major anions and cations, amino acids, glucose, protein, total and non-protein nitrogen, and brief work on lipid constituents. The pH, weight of total solids, and depression of freezing point of haemolymph are also determined together with additional work on the pO2 of haemolymph from Lymnaea stagnalis. With this information, a culture medium is devised to simulate the parasite's environment "in vitro". "In vitro" culture experiments are described starting with sterile eggs and miracidia, and the developing sporocysts are studied with the electron microscope. The "in vitro" culture of rediae dissected from snails is also attempted, with incidental observations on the maturation of cercariae. Techniques described in detail include, the large scale laboratory culture of Lymnaea truncatula, the extraction of haemolymph for analytical work, and the preparation of sterile eggs, miracidia, and rediae. Throughout the culture work, the need is stressed for criteria of growth and development "in vitro" and the comparison of this with normal growth and development "in vivo".

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INTRODUCTION

The greatest difficulties in physiological or developmental studies on most parasites result from their inaccessibility. Thus the broad aim of most attempts to culture parasites "in vitro" is to supply an artificial environment in which growth and development can easily be studied outside the host, (Dougherty, 1959). Mere survival of parasites "in vitro" is usually of very limited value. Apart from allowing easy access for many types of study, "in vitro" culture can be used to collect parasite metabolites to study their antigenic properties (Mills and Kent, 1965), to study the behaviour of parasites in immune sera (Kagan and Levine, 1956, Mueller, 1961); and to assess the effects of drugs on parasites (Schwabe, Hadidian, and Kouns, ~~et al~~, 1963). The techniques and uses of "in vitro" culture of both protozoan and helminth parasites have recently been reviewed by Taylor and Baker (1968).

The ideal approach to the production of a defined medium for "in vitro" culture would be to determine exactly the chemical and physical nature of the parasite's environment, and to reproduce this in a suitable sterile culture apparatus. This is a virtually impossible goal, but it remains the only "logical" and potentially the most successful approach, even if only partially attainable. This approach also provides useful information on many aspects of the host-parasite relationship.

Unfortunately, many workers have been dissuaded from this logical analytical approach, perhaps by the length and difficulty of the task. Thus, many complex non-defined culture media have been developed for parasites, often incorporating sera or tissue extracts totally unrelated to the parasite's natural environment. Chick embryo extract and many mammalian sera and tissue extracts have been extensively used (Smyth, 1947; 1949; 1959; Berntzen 1961, 1962). This rather "empirical" approach to the parasites environment has produced some successful culture media and suggests that in some cases the provision of a tolerable non-defined environment is better than expending time and effort in trying to simulate the "in vivo" environment. The ubiquitous nature of many biochemical systems probably accounts for much of the success of the empirical approach.

Indeed, even in the field of mammalian cell and tissue culture - from which most of the principles of "in vitro" culture of parasites derive - there exist very few examples of "cell lines" growing on logically developed, fully defined media, in spite of the present wealth of knowledge of mammalian biochemistry and physiology (Morgan, Morton and Parker et al., 1950; Healy, Fisher and Parker et al., 1955; Healy and Parker, 1966a). It is, of course, possible for a defined medium to contain protein or other substitute macromolecules (Healy and Parker, 1966b) as long as these components are themselves adequately defined i.e.

pure preparations of known molecular weight. While there are isolated examples of successful "in vitro" cultures of parasites in fully defined, protein-free media (Taylor 1963a, 1963b), it appears that most endoparasites require specific macromolecules both as chemical and physical constituents of their environment. The empirical approach adds these as a whole serum or tissue extract.

Against the empirical approach is the consideration of the reproducibility of a complex non-defined medium. Commercial serum preparations and tissue extracts are often very variable in composition, and some recommended supplements - such as a-gamma calf serum (Berntzen, 1962) - are both exspensive and difficult to obtain. Also, in the "in vitro" culture of helminth parasites, the empirical approach has only been successful developing culture media for organisms with relatively unspecialised environmental requirements. Pseudophyllid Plerocercoids (Smyth, 1959; Mueller 1959; Hopkins and Sinha, 1965) and strigeid metacercariae(Ferguson 1940; 1943; Bell and Hopkins, 1956; Wyllie, ~~et al~~ Williams, and Hopkins, 1960; Williams, ~~et al~~ Hopkins, and Wyllie, 1961; Williams, 1963), are examples of parasites which "anticipate" a change of host, and which would probably tolerate a wide range of environments, within certain limits of pH, tonicity, and availability of energy sources and macro-molecules. The successful culture of some adult

cyclophyllidean cestodes (Berntzen, 1962; Hopkins, 1967), may also be attributed to their changeable "in vivo" environment.

Thus, in the present study on larval digenetic trematodes, the logical analytical approach is used to develop a culture medium and only trace components of the medium are added empirically.

Previous attempts to culture ~~digenetic trematodes~~ Digenea "in vitro" have centered on the adult stages. This is presumably because of veterinary and medical considerations, and also because of the relatively extensive information available on the physiology and biochemistry of vertebrate hosts. The absence of any comparable information on the molluscan hosts, and the apparent complexities of larval trematode development are a little daunting when considering attempts at "in vitro" culture. It appears that the main problem in culturing adult trematodes "in vitro" is to produce an environment rich enough to supply the parasite's nutritional requirements during sexual maturation, egg production, and oviposition (Senft, 1965; Clegg, 1965). Most of the work has been, predictably, on schistosomes and this has produced some useful media and useful designs for culture apparatus (Robinson 1956; 1958; 1960; Senft, 1958; 1963; 1965; Clegg, 1959, 1961). Attempts to culture Fasciola hepatica adults (Stephenson, 1947; Dawes, 1954; Rohrbacher, 1957) and other species such as

Haplometra cylindracea (Dawes and Mueller, 1957) and Clonorchis sinensis (Hoeppli, Feng and Chu. ~~et al~~, 1938) have resulted in little more than survival of the parasites in a variety of media, although sometimes for very long periods.

Smyth (1962) suggests that the larval stages of ~~digenetic trematodes~~ Digenea should readily lend themselves to "in vitro" culture, as they occur in sites in the host which are sterile. Successful "in vitro" culture of larval trematodes would make possible many important immunity studies using cercarial and metacercarial antigens: since large amounts of material would then be easily available. Attempts to produce acquired immunity to Fasciola hepatica using whole normal or X-ray irradiated metacercariae have had very limited success. Lang (1967) reviews the work. However detailed biochemical and preparative studies on metacercarial antigens have yet to be performed. Successful "in vitro" culture would certainly make these possible.

Unfortunately the chances of successful "in vitro" culture of larval trematodes are somewhat lessened by the rather scant information available on the physiology and biochemistry of the molluscan hosts. Although the cytological and histological changes during larval development are well known for several species (Chen

Pin Dji, 1937; Rees, 1940), the host parasite relationships of larval trematodes are not well understood, and the migrations during development have never been adequately studied in terms of environmental changes. At present, the only information available on molluscan tissues as an environment derives from the analytical work on haemolymph. The more detailed analyses have been made of the haemolymph of the large terrestrial pulmonates such as Arion ater (Roach, 1963) and Cepaea nemoralis (Trams, ~~et al~~ Lowe, Bourke, and Tower, 1965). Roach (1963) discusses the previous analytical work and the attempts to develop physiological salines for mollusc tissues.

The information available suggests that there are great differences between species in haemolymph composition. Another important factor is that the water content - and thus the haemolymph composition - of the terrestrial species is known to vary considerably with changes of environment, Arvanitaki and Cardot, 1931; 1932; Kamada, 1933; Howes and Wells, 1934; Martin, ~~et al~~ Harrison, Hunton, and Stewart, 1958). Thus it is important in analytical work to remember that the concentration of haemolymph constituents will vary according to the "state of hydration" of individual snails.

There is no specific information on the composition of the haemolymph of Lymnaea truncatula presumably because of its

small size. However, Kendall and Ollerenshaw (1963) have described the effects of host nutrition on larval Fasciola hepatica. Lymnaea truncatula, being semi-terrestrial, can be expected to show wide variation in haemolymph composition depending on its physical environment and availability of food. Thus larval Fasciola hepatica have to be able to tolerate a changing environment and to adjust their metabolism accordingly. Assuming adequate availability of haemolymph for analysis, it should be possible to define an acceptable environment within this range. The most difficult tasks will probably be to supply acceptable macro-molecules and to simulate "in vitro" the changes in physical conditions between miracidial, sporocyst, and redial environments.

Indeed there has been little work towards the "in vitro" culture of larval digenes. Ingersoll (1956) describes survival of miracidia containing rediae of Cyclocoelum microstomum in a variety of media, and Friedl (1961a, 1961a) the "in vitro" survival of Fascioloides magna rediae. Chernin has done much useful ground work towards sporocyst and redia "in vitro" culture, including haemolymph analyses and organ culture of Australorbis glabratus (1963), a method for obtaining bacteriologically sterile snails (Chernin and Schork, 1959), and development of operational techniques for transplanting parasites from infected to non-infected snails (1966). Chernin (1964) also observed cercarial emergence "in vitro" from redia tissue preparations, Cheng (1963) reviews much of the work, and discusses the nutritional requirements of larval trematodes. The most impressive work, however, remains that of Barlow (1925), showing that miracidia of Fasciolopsis buski will change into young sporocysts and show limited growth and development "in vitro" in planorbid haemolymph. Similar work on the miracidia of Fascioloides magna (Campbell and Todd, 1955) and Schistosoma mansoni (Bénex and

and Lamy, 1959; Michelson, 1964; Targett and Robinson, 1964) suggest that the miracidium is the best point from which to start "in vitro" culture of larval digene.

Thus, in the present study, Fasciola hepatica and Lymnaea truncatula were chosen as the species for work towards "in vitro" culture of larval trematodes. These species can be easily obtained for laboratory work and the economic importance of Fascioliasis is an added reason for their choice. It was decided that a detailed analysis of haemolymph was needed to provide information on the environment of larval Fasciola hepatica. This in turn required the development of techniques for collection of haemolymph, and the use of ultra-micro analytical methods. The results of the haemolymph analysis lead to "in vitro" culture work and the development of a basic culture medium, with the expected attendant difficulties in developing techniques for sterile handling of organisms and criteria for assessing development "in vitro".

PART I The maintenance of *Lymnaea truncatula* in the laboratory.

Introduction

Thomas (1883) first suggested that the snail *Lymnaea truncatula* was concerned in the life-cycle of *Fasciola hepatica*, and numerous later authors have confirmed this species as the only important snail host. Kendall (1950) has shown the unsuitability of other *Lymnaea spp.* *Lymnaea truncatula* can be easily maintained in the laboratory (Taylor and Mozley, 1948), andit has now become routine laboratory practice to culture the blue-green algae *Oscillatoria spp.* as a source of food (e.g. Wilson 1965). With abundant food supply snails can be reared from eggs to fully - grown sexually mature adults in as little as 8 weeks (Kendall and Ollerenshaw, 1963).

The techniques described here allow a very large population of snails to be kept with less labour and maintenance than are required with techniques described hitherto. A very large population of snails is needed for the present work because of the small size of *Lymnaea truncatula* and the need for considerable volumes of haemolymph for analysis. Attempts to culture *Oscillatoria spp.* in agar and liquid media are first described briefly. The development of a mass culture *Oscillatoria spp.* on mud surfaces is fully described together with the maintenance of *Lymnaea truncatula* on the algal cultures.

Methods

A culture of *Oscillatoria spp.* growing on a mud surface

was obtained from the Ministry of Agriculture and Fisheries Central Veterinary Laboratory, Weybridge, Surrey.

(1) The Culture of Oscillatoria spp. in agar and liquid media.

Attempts were made to grow Oscillatoria spp. in agar and liquid media chosen with advice from the Culture Collection of Algae and Protozoa, Downing St., Cambridge. It should be stressed here that these attempts were made with a view to producing a mass culture of algae without using laborious sterile techniques to exclude contaminating micro-organisms: in short to assess the possibility of using synthetic media for rapid non-sterile algal cultures (as produced with mud surface methods). Thus the failure of some of the media used here to produce successful culture for the purposes of this study does not mean that these media would not support a pure culture of Oscillatoria spp. under axenic conditions.

Algal filaments teased from the Weybridge culture were used as inoculants for the cultures. The basic agar medium used was termed "U.C. Agar". Its composition was: KH_2PO_4, 0.1%; Na_2CO_3; 0.15%; $MgSO_4 \cdot 7H_2O$; 0.025%; NH_4Cl, 0.005%; $Ca(NO_3)_2$, 0.0025%; trace element solution, 0.1% (by volume); Agar, 1.0%. The composition of the trace element solution was, in mg per litre: $Na_2B_4O_7 \cdot 10H_2O$, 20; $CuSO_4 \cdot 5H_2O$, 100; $FePO_4$, 150; $MnSO_4 \cdot 4H_2O$, 20; $Na_2MoO_4 \cdot 2H_2O$, 10;

; $ZnSO_4 \cdot 7H_2O$, 100. The trace element solution was filtered before use, to remove a precipitate.

Using U.C. Agar as above, and increasing the $Ca(NO_3)_2$ content x 4 and x 10, numerous plates were prepared using autoclaved media and heat sterilised 3¾ inch glass petri dishes. Cultures were inoculated by surface streaking with algal filaments; watered with a thin overlay of sterile distilled water and left in daylight at room temperature.

Liquid cultures were based on soil extracts. Soil extract was made by adding a volume of soil to roughly twice its own volume of water, and heating in a steamer for 3 hours. The debris was allowed to settle and the supernatant retained and filtered when cool. This basic extract is termed "e" at the Culture Collection of Algae and Protozoa and a widely used medium based on it is "e + s" which has the following composition: soil extract "e", 10%; KNO_3, 0.02%; K_2HPO_4, 0.002%; $Mg\ SO_4 \cdot 7H_2O$, 0.002%.

"e" was prepared using the soil used at Weybridge for routine cultures - a clay soil from Somerset - and attempts were made to grow Oscillatoria spp. in "e + s" and in "e + s" with the nitrate content increased tenfold. Also cultures were set up using both these media, adding pieces of ashed filter paper to provide a substrate for algal growth. Further attempts were made using "e + s" with the "e" fraction

derived from local sand and gravel soils, increasing the amount of "e" in the media up to 100%. In all the above cultures, seeding was with algal fragments broken up by light homogenisation. Culture vessels were 100 ml and 500 ml conical flasks plugged with cotton wool. Both static and shaken cultures were tried, at room temperature under continuous lighting from fluorescent striplights. All media were sterilised by autoclaving, and all glass apparatus by dry heat.

(2) The culture of Oscillatoria spp. on mud surfaces.

Taylor and Mozley (1948) report that algae cultured to feed Lymnaea truncatula thrive only on the heavy clay soils on which the snails abound in the field, and also mention the necessity of using pond or stream water for mixing and subsequent watering of mud cultures. The original culture received from Weybridge was growing on such a clay soil from Somerset. Although it entailed a very long journey and would probably be impracticable to use it for large scale cultures, a quantity of this soil was collected for trial cultures. Also samples were taken of 13 local soils of different geological parentage and the pH of each soil determined very approximately, mixing each to a slurry with distilled-deionised water and reading on the pH meter. Table I describes the soils used.

About 1 Kgm of each of the 13 local soils was sterilised by autoclaving at 15 psi for 20 minutes. This initial sterilisation was merely to eliminate seeds and large soil organisms, and after this no attempt was made to follow sterile techniques. When cool, the soil was spread out on a polythene tray and stones and other debris removed. At first attempts were made to grade and break up the soil using a mechanical mill and sieves, but these procedures produced no benefits, and acceptable mud media were prepared without them, saving much time. Each soil was mixed and kneaded by hand to a stiff mud, adding small amounts of well-aerated tap-water. Tap-water bubbled vigorously with air for at least 7 days contains no harmful chlorine. It is preferable to pond water for mixing mud media as it contains fewer micro-organisms to contaminate the cultures, and is easily obtained in large quantities. It appears that both snails and algae deteriorate if distilled water is used for mixing and watering cultures (Wilson, 1966, personal communication).

Mud surfaces were prepared by compressing the mud into 10 inch square transparent plastic dishes to give a level layer of mud about $\frac{1}{2}$ an inch deep. No depressions were made in the mud surface (Taylor and Mozley, 1948). Plates 1 to 3 illustrate the preparation of mud surfaces. (see below). Two culture dishes were prepared for each of the 14 types of soil and these were seeded by smearing the entire surface with algal fragments, scraped from the original Weybridge culture by using a spatula.

After watering with a thin overlay of aerated tap water, the cultures were covered with glass plates and held in a Dexion frame under fluorescent striplighting. The lights, 5 foot tubes of Crompton 65 watt fluorescent and Philips 80 watt "cool white" were set 3 feet above the dishes and were controlled by a time switch set to give 12 hours darkness and 12 hours light. Room temperature remained around 20°C, and accurate records were kept using a thermograph. The dishes were watered daily for 7 days, to maintain the aerated tap water overlay, and then removed from the frame and assessed for growth. Table I gives the results.

Using the results of these first trials, and some empirical trials adding inorganic salts to mud media, a large-scale culture procedure was developed. The soil chosen for this was the Millington chalk soil (Table I). Empirical trials showed that the addition of K^+, Ca^{++}, NO_3^- and various phosphates greatly accelerated the growth of Oscillatoria spp. on the mud surfaces. For the large-scale culture, solid calcium nitrate and potassium di-hydrogen orthophosphate were added to the cooled soil after autoclaving, in quantities calculated to give a final concentration of 0.5 gm of each per dish. The soil was then thoroughly mixed before mud preparation. Normally 20 to 25 kgm of soil were used, and autoclaved in a galvanised bin. All procedures for preparing the dishes was as described above and in Plates 1 to 3. For this large-scale work, the light control switch was removed and cultures illuminated continuously for 7 days. 20 to 25 kgm of soil was sufficient to make between 40 and 50 culture dishes.

(3) Maintenance of Lymnaea truncatula on mud surface algal cultures

A small population of snails was obtained with the original algal culture from Weybridge. The snails were kept on the mud surface algal cultures at room temperature on darkened shelves. No additional foods were added (Taylor and Mozley, 1948). Mud dishes supporting snails were watered daily as for the algal cultures. A population of several thousand snails was then built up. The snails deposited large numbers of egg masses, mainly in the corners of the dishes. These were collected at intervals and kept in petri dishes containing aerated tap water, for up to three weeks. The eggs hatched at various times over the 3 week period, being of different ages. The minute hatched snails were transferred directly to mud surface algal cultures using a camel hair brush. Several hundred were placed on each dish, and 3 or 4 such dishes prepared every week.

After about 10 days, the young snails were transferred to fresh algal cultures in smaller numbers, being large enough to be handled with fine forceps without damage. Thereafter snails were transferred to fresh cultures every week, reducing the numbers per culture as their food requirements grew. Thus a typical mud surface culture would support 100 snails of total shell length 4 mm for one week, or 30 snails of total shell length 7 to 8 mm for a similar period.

Results

(1) The culture of Oscillatoria spp. in agar and liquid media

Agar based-cultures

The Oscillatoria spp. on basic U.C. agar appear to grow (or spread?) very fast for 3 to 4 hours after seeding. The algal filaments spread all over the surface of the agar and some were seen to penetrate down into the medium. After about 5 hours, however, no further development occurred. It is probable that this initial phase was merely locomotion of algal filaments giving an illusion of growth. Increasing the nitrate content of the medium produced no better results. In all cultures severe contamination with other micro-organisms was seen after 24 hours, suggesting that a sterile inoculum is essential for agar-based cultures. It was apparent that agar-based cultures would not support any useful growth of Oscillatoria spp. for the purposes of feeding snails without recourse to careful sterile technique and extensive work to find a suitable medium.

Soil extract cultures

None of the soil extract cultures produced anything more than a thin film of alga lining the surface of the culture vessel and filter paper substrates, where present. In all cultures contamination with other micro-organisms was found to be severe after 5 days, and it was apparent that liquid cultures of Oscillatoria spp. were of no use for the purposes of feeding snails. One interesting observation, again

illustrating the locomotion of algal filaments, was the homogenised algal filaments aggregated to form a compact clump in static cultures. This occurred within a few hours of seeding with the homogenised suspension. The same phenomenon was observed in shaken cultures if shaking was discontinued.

(2) The culture of Oscillatoria spp. on mud surfaces

The Somerset clay soil produced very poor results and is not included with the 13 local soils in Table I (below). Table I scores the soils from 1 to 13 according to the growth of Oscillatoria spp. they supported after 7 days culture.

Table 1 - Summary of soil data

Soil(Geological Parentage)	Grid Reference (O.S. Sheet 98)	pH	Scored
Chalk (Millington)	795630	8.0	1
Sand and gravel	704558	7.3	2
Lower oolite	750629	7.5	3
Sand and gravel	705554	6.8	4
Rhetic beds	733625	8.2	5
Alluvium	725576	7.0	6
Lower lias clay	751625	6.9	7
Kimmeridge clay	790631	8.0	8
Keuper marl.	725613	7.8	9
Lower lias clay	757633	6.7	10
Sand and gravel	652518	6.8	11
Oxford clay	784633	7.7	12
Boulder clay	682544	7.2	13

* Soils are scored from 1 to 13 in order of proficiency in supporting growth of Oscillatoria spp.

The chalk soil, from Millington Pastures, near Pocklington, East Riding of Yorkshire, was by far the best soil for mud surface culture work. Collection of this soil was normally by taking the fine chalky topsoil, thrown up as molehills. This was largely free from stones and debris, and was ideal for mixing mud. An interesting incidental observation was that this locality supports a large population of Lymnaea truncatula, indicating that the soil has an acceptable pH and chemical composition for keeping snails, as well as for growing the algae on which they feed.

Using the Millington Chalk soil and the large scale culture procedure, a thick matt covering of Oscillatoria spp. was grown on each culture dish within 7 days. Plates 1 to 3 illustrate this and the soil they depict is Millington Chalk soil with the additional inorganic salts. This large scale culture procedure was a complete success and was adopted as a weekly routine, requiring about 5 man-hours per week to produce and tend 50 culture dishes. In general, contamination of cultures presented no problems, but did occasionally occur in the form of other algae and bacteria. Contamination did occur when less chalky soil from thebottom of the valley system at Millington was used. This darker soil favoured the growth of contaminants and this, together with the failure of the Somerset soil found to be successful at

Weybridge, shows that soil types can vary considerably within a very small area. It is also suggested that addition of inorganic salts in amounts of more than 0.5 gm per culture dish also favoured the growth of contaminants. With the correct Millington Chalk soil the Oscillatoria spp. spread and grew so fast as to occupy the whole of the mud surface ahead of any contaminants.

(3) Maintenance of Lymnaea truncatula on mud surface algal cultures

Snails grew and developed very quickly on the algal cultures and produced large numbers of egg masses enabling a large population to be built up very quickly. Snails were reared from eggs to egg-producing adults in under 7 weeks. Adult snails always appeared large and healthy frequently attaining a total shell length of 8 mm. A minority of snails on the culture dishes tended to climb up the side walls of the dishes and to go into temporary aestivation. This became a serious problem only if the mud surface was allowed to become dry or if the cultures became at all contaminated. In general the open nature of the cultures (lack of cover) had no deleterious effects on the snails, and was only shown by their preference for depositing egg masses in the corners of the dishes.

The snails, in contrast to most aquatic Lymnaeidae, were easily maintained free from parasites and disease. The only pathogen noticed was a fungus which did kill a number of snails. This appeared as a furze of fungal hyphae on the shells of infected snails, which proliferated greatly over their entire bodies and on the mud surface nearby after death. It is possible that this was a secondary saprophytic fungus, and not the main cause of death. Generally, with the exception of the newly hatched snails - which were easily damaged during transfer -, mortalities were very low. High mortalities in larger snails occurred on one occasion when the temperature of the culture room rose to 25°C. At 18 to 20°C, with dishes carefully prepared and tended, mortalities were negligible.

Discussion

The failure of the culture attempts using agar and liquid media was disappointing, but the success of the large-scale mud surface cultures, without recourse to any sterile technique was very satisfactory. The method described is a vast improvement on Taylor and Mozley's method (1948), as their cultures took up to 3 weeks to produce enough algae to support a population of snails. Also the addition of inorganic salts to the soil used, gives a much faster growth rate and a thicker algal matt than in the original culture obtained from Weybridge. The Millington location is a very reliable

source of soil, being on common land, although collection of frozen soil during the winter months proved very laborious,

The methods described do allow a large population of snails to be kept with a realistic expediture of time and effort. A scheme was devised that would supply up to 300 adult snails per week, requiring 10 to 12 man-hours labour per week, including the time for preparing the algal cultures. The scheme was not altogether successful for the present study, as the demand for snails varied from nil to "as many as possible" per week as new analytical methods were tested (Part III). However the system described, and the rapid breeding cycle of Lymnaea truncatula allow the population to be built up or run down as required. The cultures of snails used throughout the present study show much quicker development than recorded by other authors (Taylor and Mozley, 1948; Kendall and Ollerenshaw, 1963).

Plate 1

a) and b) Preparing a dish for mud surface culture.

a)

b)

PLATE 1

Plate 2

a) Scraping alga from an established culture for seeding purposes.

b) Seeding a new mud surface culture.

a)

b)

PLATE 2

Plate 3

a) A comparison between an unseeded dish and a normally seeded dish both left for 7 days, showing the good growth of Oscillatoria spp. and the absence of any contamination.

b) A "spent" culture, having supported 40 adult snails for 7 days, showing that nearly all the cultured alga is acceptable food.

a)

b)

PLATE 3

PART II The collection of haemolymph for subsequent analysis.

Introduction

Most of the difficulties in an analysis of the haemolymph of Lymnaea truncatula arise from the small size of the snails. Although no work was done to determine actual blood volumes (Cheng, 1963), it was estimated that only 1 or 2 µl of haemolymph would be obtainable from each snail. In previous analytical work on larger species, collection of haemolymph has been by draining either the heart or haemocoele. Roach (1963) and Trams et al (1965) are good examples and summarise the previous work. The large species of snails used in previous work, such as Lymnaea stagnalis, Arion ater, and Helix spp., are all capable of providing a sample of between 0.5 and 1.0 ml of haemolymph per individual.

Picken (1937) collected haemolymph from the heart and haemocoele of Lymnaea peregra, the former samples being around 1µl in volume. The main problem with collection from the heart is that the pericardium must first be emptied before puncturing the heart proper. Picken (1937) and Potts (1954) achieved this with both Anodonta sp. and the large Lymnaea spp. The difficulties in repeating their work using Lymnaea truncatula are very great, but some brief attempts to collect haemolymph from the heart and major blood vessels are described.

Collection of haemolymph from the haemocoele of Lymnaea truncatula is also fraught with difficulties. The head-foot of the snail contracts immediately when manipulated and collection from the contracted head-foot is impossible. The base of the contracted head-foot forms a formidable barrier of dense tissue and mucus that would block any micropipette traversing it. Also, in this contracted state, the accessible haemocoele around the gut contains very little haemolymph anyway. In short, the problem is that the shell itself, and contraction following manipulation, prevent access to haemocoele. De-shelling the snail is not a satisfactory answer as it would be far too time - consuming for collection on a large scale. Drainage of haemocoele by general puncture of the shell spire is possible, but this provides a sample contaminated with other tissue fluids.

The answer to the problem is provided by anaesthetisation techniques. Lever, ~~et al~~ Jager, and Westerveld. (1964) describe a rapid method of anaesthetisation for Lymnaea stagnalis, a fully aquatic species. This method is shown here to be equally effective with the more terrestrial Lymnaea truncatula, but subsequent experiments suggest that it may provide an unreliable haemolymph sample. An alternative method of anaesthetisation more suitable for Lymnaea truncatula is fully described, and the effects of anaesthetisation on the snail are discussed. With accessible

haemocoele, collections of haemolymph samples is relatively easy. Methods of collection, pooling, and storage of samples for future analytical work are fully described.

Methods

(1) Collection of haemolymph from the heart and major vessels

The snail was held in a small perspex clamp mounted on the micromanipulator. The clamp spring mechanism was constructed from parts taken from a microscope stage slide-holder. With low power magnification (dissecting microscope) the heart and major circulatory vessels were easily visible through the translucent shell. Attempts were then made to remove the area of shell above the heart.

Attempts to soften and remove shell using small spots of dilute mineral acids were complete failures and invariably killed the snail. The successful method employed a fine drill. The instrument used was battery-powered, trade name "Minidrill", fitted with a 0.0135 inch bit (Dormer 70). This drill operated at 8,700 r.p.m. with 14.5 volts applied. The clamped snail was positioned using the micromanipulator, and a hole drilled in shell above the heart, racking the snail up to meet the drill. The drill was clamped

vertically during the procedure, and the heartbeat of the snail was observed throughout the procedure.

With the pericardium and heart now accessible, attempts were made to introduce glass micropipettes into the latter. These were very fine glass capillaries, made by drawing-out 1 µl capacity Shandon microcapillaries in a microbunsen flame. A single micropipette was mounted on a second micromanipulator. Results of attempts to penetrate the heart and major blood vessels are given below.

(2) <u>Collection of haemolymph from haemocoele: anaesthetisation of snails in liquid media.</u>

(a) <u>The anaesthetisation process</u>

The method used was that of Lever et al (1964). Three 1 litre beakers were held at 24°C in a water bath, two of them containing 500 ml each of a 0.1% aqueous solution of sodium pentabarbitone, and the third 500 ml of an aqueous solution containing 0.1% sodium pentabarbitone and 0.1% M.S. 222 (Sandoz). The latter compound is a well-known anaesthetic for cold-blooded vertebrates and invertebrates (e.g. Mcgovern and Rugh, 1944; Randall, 1962). All solutions were

made up in distilled-deionised water. The first solution of 0.1% sodium pentabarbitone was de-oxygenated by rapid bubbling of nitrogen from a porous stone for at least 10 minutes.

Active snails, taken from flourishing algal dishes, were anaesthetised in batches of 30. The snails were placed in a perforated polythene jar, fitted with a wire handle. This allowed easy transfer between solutions without manipulating the snails. The snails were first immersed in the de-oxygenated 0.1% sodium pentabarbitone solution for exactly 5 minutes. During the period, the beaker was covered with a glass plate, and nitrogen bubbled slowly to maintain the oxygen deficit. Any snails floating on the surface of the solution were freed from gas bubbles and sunk using forceps.

The snails were then transferred to the second 0.1% sodium pentabarbitone solution, and carbon dioxide gas bubbled vigorously through the solution from a porous stone. The snails were kept in this solution for a further 5 minute period. The porous stone was kept outside the polythene container housing the snails, but fine bubbles of carbon dioxide passed through the perforation and circulated around the snails. Finally the snails were transferred to the solution of sodium pentabarbitone and M.S. 222 for a 5 minute period.

Recovering from anaesthesia was tested by immersing the snails in aerated tap-water at 24°C and observing their behaviour. Snails were immersed for exactly 1 hour, and then returned to algal dishes where mortalities were recorded over a 5 day period. Three batches of 30 snails were tested for recovery from anaesthesia.

(b) The effect of anaesthesia on the water content of the snail.

Experiment 1. A comparison of the fresh and dry weights of anaesthetised and control snails.

The water content, and hence the fresh weight, of terrestrial snails is notoriously variable (see Discussion below). It was felt that a comparison of the water content of groups of anaesthetised and control snails would show if the snails became significantly hydrated during the anaesthetisation process. Snails were taken from the same algal dish, ensuring that they were all initially in about the same state of hydration. 10 snails were anaesthetised as above, and 10 control snails immersed in aerated tap-water for the 15 minute period. This provides a better control than taking snails directly from the algal dishes as it takes into account excess water that may become trapped between shell and head-foot when snails are immersed in solutions. It is also a perfectly valid control as Lymnaea truncatula will survive in water for long periods of time without apparent osmotic stress and therefore presumably with no significant

dilution of the haemolymph. The snails used here were all of total shell length 6 mm ± 0.1 mm, measured with vernier slide calipers.

The anaesthethised and control snails were blotted dry and placed singly on weighed discs of aluminium foil, which were then placed in a humidity chamber to prevent desiccation during weighing. The latter was made by filling the base of a dry-seal glass desiccator with moist cotton-wool. Each snail and foil piece was weighed and groups of weighed snails placed in covered glass.crystallising dishes. The snails were dried at 105°C for 24 hours in a hot box oven and then allowed to cool in a desiccator. The dried snails were then re-weighed. Re-drying and weighing to a constant weight was not considered necessary for such small specimens. All weighing was performed on the 0 to 50 mg scale on the Cahn gram-electrobalance.

Experiment 2 A comparison of the fresh weights of anaesthetised and control snails, dissected from their shells.

Selection of snails, and experimental and control treatments were as in experiment 1 above, using 20 anaesthetised and 20 control snails. Snails were taken singly and dissected free from shell, blotted dry, and weighed on aluminium foil discs of known weight. Dissection was by first gently crushing the shell by applying a rounded glass rod to the dorsal surface of the largest shell whorl. Then shell fragments were broken and picked away

using fine dissecting needles. If any damage was noticed to the snail tissue, either during dissection or blotting dry, the snail was discarded. All dissections were performed under water. Weighing was performed as in experiment 1. above.

Experiment 3 A comparison of the dry weights of anaesthetised and control snails, to assess loss of ions during anaesthetisation

The snails used in experiments 1 and 2 were selected and measured very carefully - total shell length 6 mm $\pm$ 0.1 mm - but the results show that there is considerable variation in both fresh and dry weights within the experimental and control groups (see Results, Tables 2 and 3). A preliminary experiment was performed to see if this variation could be reduced by the initial selection of snails being made more rigid i.e. selecting on size, shape and fresh weight. 15 control snails were selected from the same algal culture each having total shell length 6 mm $\pm$ 0.1. mm; and having a fresh weight between 28 and 30 mg. All the snails were of similar shape. The snails were dissected from their shell and dry weights determined as in experiments 1 and 2. In a further effect to reduce variation, 100 snails were selected as above and 50 anaesthetised and 50 control snails used for dry weight comparison. Here the snails were smaller, total shell length being 5 mm $\pm$ 0.1 mm, and dissection from the

shell was omitted. Weighing was performed on the 0 to 20 mg scale of the Cahn gram-electrobalance.

(3) Collection of haemolymph from haemocoele: anaesthetisation of snails in gaseous media

The organic solvents diethyl ether and chloroform have been used extensively as anaesthetics, and Huf (1934) describes their use with Lymnaea stagnalis. A method was devised here for anaesthetising the more terrestrial Lymnaea truncatula using ether vapour and carbon dioxide gas, avoiding immersion in solutions. The snails surface covering of mucus in the gaseous medium prevents excessive water flux and dilution of the haemolymph. This, and possible ionic changes are discussed later. A less successful method using nitrogen instead of carbon dioxide was also developed for use when collecting haemolymph for analyses in which the acidity of carbon dioxide would introduce important error.

The snails were processed in batches of 25, being first placed on damp filter paper in a glass crystallising dish and left for at least 15 minutes to extend and recover from manipulation, when removed from the algal dishes. Snails remaining contracted or inactive after this period were discarded. The crystallising dish was then placed in a humidity chamber. This was, in fact a Jencons

12 inch diameter dry-seal safety desiccator, with the base portion filled with moist cotton wool. The lid of the dessicator was fitted with a rubber bung, bored to allow gases to be blown through the chamber. The snails were first exposed to ether vapour for between 10 and 15 minutes by placing a 100 ml beaker full of ether adjacent to the dish containing the snails, and then closing the dessicator. Leaving the ether beaker in position, carbon dioxide or nitrogen was then blown through the chamber to complete the anaesthetisation, the former for a minimum of 15 minutes, the latter for a minimum of 25 minutes.

Recovery from anaesthesia was tested both by returning snails directly to the algal dishes, and by placing snails in aerated tap-water, as following anaesthesia in liquid media. Mortalities were recorded over a 5 day period. Incidental observations were made on the appearance and recovery of snails exposed to the anaesthetics for longer periods of time.

(4) <u>Collection of haemolymph from haemocoele: collection, pooling and storage procedures</u>

A brief histological examination of the head-foot of

anaesthetised snails showed the presence of very large haemocoele around the anterior gut. Serial sections of head-foot were cut from paraffin-wax embedded blocks. This haemocoele has been used by many authors for haemolymph collection (e.g. Picken, 1937; Roach, 1963). The method of collection used in the present study involved insertion of a fine micropipette through the dorsal head-foot wall and into the haemocoele surrounding the gut, just posterior to the buccal mass. This avoided touching any internal organs or ducts.

Snails used for haemolymph collection were large, active individuals, and anaesthetisation was always by the gaseous methods. The snails were taken singly from the anaesthetisation chamber. The anaesthetised snail was held firmly in the fully extended state (Figure 1, Plate 4). The base of the head-foot was applied to the strip of filter paper (Whatman No. 1) and the shell pulled gently backwards and pressed into the plasticine. The exposed dorsal surface of the head-foot was cleaned and dried with tissue paper before inserting the micropipette. The micropipette was mounted in a Shandon microcapillary holder with a mouth-tube attached, the whole system being clamped to a micromanipulator. Viewing with a dissecting microscope, the micropipette was quickly positioned above the snail and rapidly inserted (Figure 1). Great care was taken not to insert the micropipette too far.

The spontaneous flow of haemolymph, drawn into the micropipette by capillary attraction, was observed with the microscope. When this flow had stopped, the micropipette was withdrawn very slowly, ceasing withdrawal if flow started again. After withdrawal, the snail was discarded, and the micropipette racked away using the micromanipulator. Its contents were quickly emptied into a storage tube, by means of the mouth tube. The storage tubes (Plate 4) were constructed to serve both as storage vessels and micro-centrifuge tubes, to enable immediate sedimentation of haemolymph cells and any tissue debris. They were made by suspending 2 x $\frac{1}{4}$ inch glass ignition tubes, supported by a wire loop round the rim, in a fully aerated bunsen flame. The base of the tube was held just above the apex of the blue cone in the flame, and the glass became semi-molten to form a drop-like base portion and conical interior. (Plate 4). After cooling, the distorted rim portion and upper tube were trimmed off using a diamond pencil and white hot fine glass rod.

Originally, the micropipettes were made by drawing out 1, 2 or 5 μl capacity Shandon microcapillaries in a micro-bunsen flame, constructed from a fine steel hypodermic needle. For routine haemolymph collection, however, micropipettes were made by drawing out lengths of soda glass, cut from "longform" pasteur

pipettes, to form very fine, extremely long capillary lengths. For this, an ordinary bunsen flame was sufficient. Often, up to a yard of capillary tubing finer than 1 μl capacity Shandon microcapillaries was obtained. Lengths of this capillary tubing were broken off to provide a fresh micropipette for each snail.

The procedure for pooling haemolymph samples from a batch of snails anaesthetised using ether and carbon dioxide was as follows. The 25 snails were observed closely during anaesthetisation, and those that appeared to succumb first were taken, in turn, for haemolymph collection after 30 minutes total time in the chamber. All collection from the batch was then performed during the next 15 minutes. During this time, the snails not in use were kept in the closed chamber, the lid being only partially opened to remove snails, and gas flow being maintained throughout. Thus no snail was kept for more than 45 minutes in the chamber. The short time available meant that collection procedure had to be accomplished rapidly.

During the pooling of samples, the storage tube was capped with parafilm (Plate 4). Pooled haemolymph was centrifuged for 15 minutes at mark 9 on the Griffin/Christ SS3300 centrifuge (at

approx. 3,000 r.p.m.) and the supernatant immediately transferred to a fresh storage tube using a Shandon microcapillary of known volume. Refrigeration was not thought to be necessary during the short time involved in collection, pooling, and centrifugation. Centrifuged haemolymph was either used immediately for analytical work or was stored in sealed storage tubes at $4^{o}C$ for a maximum of 2 days. For certain inorganic analyses, haemolymph was stored in sealed storage tubes at - $25^{o}C$ for up to 2 weeks.

Results

(1) Collection of haemolymph from the heart and major vessels.

Using the fine drill, a neat hole could be successfully drilled in the shell above the heart, without affecting the heartbeat or damaging internal organs. Insertion of the micropipette, however, proved almost impossible. The heart stopped beating and contracted as soon as the pericardium was ruptured. Authors working with the larger species (e.g. Picken, 1937) do not mention this. Even when the heart was punctured with certainty, the haemolymph sample obtained was contaminated with pericardial and extra-pallial fluids. This was because the extremely strong capillary attraction of the micropipettes made it impossible to hold them empty until inside the heart proper. Such fine pipettes cannot be adequately controlled by mouth tubes or rubber teat devices. A sample of about 0.1 μl of haemolymph was obtained on several occasions, but was always contaminated with other fluids.

Attempts to penetrate the major circulatory vessels were equally unfruitful, encountering the pipetting difficulties mentioned above. It was demonstrated, however, that Lymnaea truncatula can be dissected free from its shell, to allow, access to major circulatory vessels, and the heart of the naked snail will continue to beat for a considerable time. It was apparent that

attempts to collect haemolymph by dissection under oil (Trams et al, 1964) would also suffer from the problem of contamination of haemolymph samples with other fluids.

(2) Collection of Haemolymph from haemocoele: anaesthetisation of snails in liquid media.

(a) The anaesthetisation process

Complete anaesthesia of the snails was achieved. The snails extended in the first de-oxygenated solution, exposing a large area of head-foot to the anaesthetic. This provided enough anaesthesia to stop contraction when treated with the carbon dioxide and subsequent solutions. After the 15 minute treatment, the snails remained fully extended with the head-foot hanging limp from the shell. They showed no contraction when manipulated and did not appear to be swollen by the process.

Recovery in aerated tap water was always 100% successful, usually taking from 40 to 60 minutes. After this time, the snails were fully active, and contracted the head-foot when manipulated. In the 3 batches tested, normal feeding was observed throughout the 5 day period and no mortalities were recorded.

(b) The effect of anaesthesia on the water content of the snail

Experiment 1

Table 2 gives the fresh weight, dry weight, and water content of anaesthetised and control snails. All weights are expressed

in mgm. The water content is also expressed as a percentage of fresh weight.

Table 2 - The water content of anaesthetised and control snails in experiment 1.

Anaesthetised				Controls			
Fresh Wt.	Dry Wt.	Water	% Water	Fresh Wt.	Dry Wt.	Water	% Water
36.38	10.18	26.20	72.0	36.36	12.89	23.47	64.5
35.79	11.41	24.38	68.1	29.29	10.79	18.50	63.2
27.91	9.15	18.76	67.2	28.94	10.28	18.66	64.5
31.32	10.02	21.30	68.0	25.94	8.86	17.08	65.8
37.09	11.29	25.80	69.6	30.84	10.52	20.32	65.9
31.21	10.08	21.13	67.7	21.17	8.37	12.80	60.5
32.63	10.37	22.26	68.2	27.79	9.69	18.10	65.1
35.47	10.92	24.55	69.2	32.63	11.69	20.94	64.2
27.13	9.19	17.94	66.1	25.50	8.18	17.32	67.9
29.48	9.31	20.17	68.4	31.33	11.75	19.58	62.5

Analysis of data

	Anaesthised		Controls	
	Mean	Variance	Mean	Variance
Fresh Wt.	32.44	13.091	29.00	17.850
Dry Wt.	10.19	0.685	10.30	2.411
Water	22.24	8.317	18.68	7.878
% Water	68.5	-	64.4	-

The table is given in full to illustrate the large variance in fresh weight within the groups. The difference between the means of percentage water content cannot be tested statistically as the original figures are ratios. For the water content data, the difference

between the means is 3.56, and the variance ratio is 1.06. Student's t = 2.800, giving p between 0.01 and 0.02. For the fresh weight data, the difference between the means is 3.44, the variance ratio is 1.36. Student's t = 1.958, giving p between 0.10 and 0.05. The dry weight data suggest that there is no significant loss of salts during anaesthetisation but this should be tested further using less variable material and a more sensitive weighing scale. The general result is that there is a significant intake of water by the snails during anaesthetisation and with less variable material this should be demonstable simply by comparing the fresh weights of anaesthetised and control snails.

Experiment 2

Table 3 gives the fresh weights of control and anaesthetised snails dissected free from shell. The dissections of control snails, which contracted, were relatively easy. The anaesthetised snails, however, proved very delicate and difficult to dissect, the digestive gland and kidney being very easily damaged. A total of 100 snails were anaesthetised before the 20 successful dissections were performed. All weights are given in mg.

Table 3 - The fresh weights of anaesthetised and control snails in experiment 2.

Anaesthetised				Controls			
Snail	Fresh Wt.	Snail	Fresh Wt.	Snail	Fresh Wt.	Snail	Fresh Wt.
1	22.59	11	22.24	1	17.92	11	17.87
2	22.17	12	21.69	2	19.60	12	18.31
3	20.96	13	22.88	3	20.58	13	17.57
4	19.62	14	14.93	4	17.50	14	17.01
5	21.86	15	18.40	5	18.76	15	15.34
6	19.08	16	19.06	6	14.54	16	15.03
7	19.15	17	14.94	7	18.75	17	18.74
8	21.08	18	21.85	8	18.16	18	18.98
9	21.93	19	20.59	9	19.67	19	16.95
10	18.97	20	17.00	10	19.24	20	15.88

Analysis of data

Anaesthetised		Controls	
Mean =	20.05	Mean =	17.67
Variance =	5.64	Variance =	2.96

The variance ratio is 1.91 and the difference between the means 2.38 mg student's 't' = 3.639, showing that the difference is significant ($p < 0.01$). This result confirms the significant uptake of water demonstrated in experiment 1.

Experiment 3

Table 4a gives the dry weights of 15 dissected control snails, showing the variation found. The variance is low, but around 100 snails

were measured and weighed to provide the 15 selected for dry weight determinations. Table 4b gives the results of the experiment determining the dry weights of 50 anaesthetised and 50 control snails. All weights are in mg.

(a) Table 4 The dry weights of anaesthetised and control snails in experiment 3

Snail	Dry Wt.	Snail	Dry Wt.	Snail	Dry Wt.
1	9.98	6	9.42	11	10.23
2	9.38	7	9.02	12	9.08
3	9.29	8	9.59	13	10.15
4	9.86	9	8.70	14	9.55
5	8.90	10	9.93	15	9.35

Analysis of data

Mean = 9.50 Variance = 0.215

(b)

Anaesthetised				Controls			
Snail	Dry Wt.	Snail	Dry Wt.	Snail	Dry Wt.	Snail	Dry Wt.
1	8.39	26	5.63	1	6.70	26	7.20
2	7.27	27	5.64	2	8.27	27	5.74
3	5.89	28	5.50	3	7.75	28	6.64
4	8.14	29	5.59	4	7.74	29	7.56
5	7.25	30	6.82	5	6.16	30	7.22
6	7.22	31	7.18	6	7.51	31	8.11
7	6.06	32	6.87	7	5.52	32	8.27
8	7.67	33	7.34	8	7.88	33	6.31
9	7.39	34	5.57	9	6.47	34	7.50
10	7.07	35	5.13	10	5.61	35	6.59
11	7.88	36	5.90	11	5.04	36	8.61

Table 4 Cont'd.

Analysis of data

(b)

Anaesthetised				Control			
Snail	Dry Wt.	Snail	Dry Wt.	Snail	Dry Wt.	Snail	Dry Wt.
12	7.40	37	5.38	12	6.98	37	7.38
13	8.55	38	6.76	13	7.27	38	6.13
14	5.76	39	7.09	14	6.96	39	5.75
15	6.35	40	5.43	15	8.05	40	8.01
16	6.07	41	8.27	16	7.58	41	5.65
17	6.13	42	6.30	17	6.84	42	7.58
18	7.41	43	7.50	18	7.43	43	6.84
19	7.27	44	6.60	19	7.68	44	7.43
20	6.17	45	6.61	20	7.29	45	6.28
21	6.66	46	6.04	21	6.40	46	6.42
22	7.06	47	5.80	22	7.14	47	7.15
23	6.97	48	5.51	23	8.18	48	6.24
24	5.81	49	6.07	24	5.58	49	5.80
25	7.24	50	7.01	25	5.16	50	6.65

Analysis of data

Anaesthetised	Controls
Mean = 6.65	Mean = 6.93
Variance= 0.770	Variance = 0.802

The variance ratio is 1.04 and the difference between the Means is 0.28 mg. Assuming the sample are normally distributed, d = 1.582, and this shows that the difference between the means is not significant

($p > 0.10$). This and the results of experiment 1. above suggest that there is no significant loss of ions during anaesthetisation, but the variances in these experiments are relatively high and may have masked what could be only a minute change in weight.

(3) Collection of haemolymph from haemocoele: anaesthetisation of snails in gaseous media.

Complete anaesthesia was achieved and the methods described were fully satisfactory. 100% recovery of snails was observed after only a few minutes when they were either returned to the algal dishes or kept in aerated tap water. In many cases locomotion started almost immediately, especially when nitrogen was used during anaesthetisation. Three batches of snails anaesthetised by both gases showed 100% recovery, and no mortalities were recorded over a 5 day period. The carbon dioxide method produced a deeper anaesthesia than the nitrogen method and was judged to be more suitable for preparing snails for haemolymph collection. The use of carbon dioxide allowed about 3 to 5 minutes after removal from the chamber, before the snails showed signs of recovery. Snails anaesthetised using nitrogen were more difficult to manipulate, and recovered their powers of contraction very rapidly. It was thought that both methods allowed sufficient time for haemolymph collection, but that the use of carbon dioxide gave more time for careful operational technique.

Snails left in the anaesthetisation chamber for far more than

a total of 45 minutes began to secrete abnormal amounts of mucus, and after 60 minutes appeared shrunken and grotesque. Even these snails, however, recovered rapidly when returned to the atmosphere or to aerated tap water, normally commencing locomotion after about 15 minutes. One disturbing observation was that carbon dioxide appeared to soften the snails shell, presumably by its acidic action. The effects of this and of mucus production on the composition of haemolymph are discussed below.

(4) Collection of haemolymph from haemocoele: collection, pooling, and storage procedures

The method described was fully satisfactory, allowing from 1 to 3 μl of haemolymph to be collected from each snail. With practise, the time required to collect from each snail was reduced to about 30 seconds. This technique was rapid enough even for snails anaesthetised using nitrogen. The above volumes were estimated during the early work using drawn-out Shandon microcapillaries of known volume. The very fine, long micropipettes drawn from pasteur pipette glass were far better for haemolymph collection, and required no further drawing out to a finer point. It was found that any micropipette used had to have a sharp jagged point. This was because snail tissue is extremely plastic, and rounded or square-cut capillaries either merely spread

out the soft tissue, or required so much pressure that they penetrated in an uncontrollable manner.

The micro-manipulator allowed very speedy and accurate positioning of the micropipettes. The main difficulty with the micropipettes was mucus contaminating their tips. This presumably occurred during the slow withdrawal from the animal. Even a small amount of mucus blocked the micropipette and made it useless for work with other snails. Because of this, the rule of a fresh micropipette for each snail was rigidly adhered to. The large numbers of micropipettes required provided another reason for abandoning the use of Shandon microcapillaries, as these are significantly expensive when used in quantity, compared to homemade capillaries of negligible cost.

Figure 1 and Plate 4 show how the rim of the shell can be used to compress more haemolymph into the anterior haemocoele when extending the snail. This additional procedure was not essential, but it often provided a useful positive pressure to assist the capillary attraction of the micropipette, and sometimes allowed a larger volume of haemolymph to be collected. With practise it was possible to collect and pool at least 20 μl of haemolymph within the 15 minute period allowed by the anaesthetisation process. The storage tubes were very easy to construct and were of ideal size (total capacity about 0.1 ml). It deserves mention that the shape of a storage tube could not be altered after it had cooled down after initial manufacture. Attempts to modify

cooled tubes invariably resulted in the base portion shattering, sometimes dangerously. This was presumably due to the low quality glass used for ignition tubes, and the lack of annealing.

Discussion

The work shows that the well tried methods of haemolymph collection cannot be used with Lymnaea truncatula, mainly because of its small size. The use of anaesthetisation has many drawbacks, and willl undoubtedly affect the composition of haemolymph to some extent. Huf (1934) describes water flux and ionic changes in anaesthetised Lymnaea stagnalis, and Lever et al (1964) record a similar uptake of water. Indeed some dissection guides recommend extreme anaesthetisation of mollusca to produce a turgid condition facilitating easy dissection. The experiments performed here show that the method of Lever et al (1964) cannot be used for the purposes of the present study as it causes hydration of Lymnaea truncatula and hence dilution of haemolymph.

Unfortunately there appears to be no alternative to using some method of anaesthetisation. Previous workers with aquatic species have used compounds such as menthol and chloralhydrate (Van Eeden, 1958) and nembutal and alcohol (Van der Schalle, 1957), but these methods require immersion of the snails in solutions for long periods of time and would almost certainly produce worse hydration of Lymnaea truncatula than the rapid method tried (Lever et al, 1964). The

gaseous methods of anaesthetisation offer the only possible solution.

The greatest advantage of the gaseous methods developed here is the rapid recovery of the anaesthetised snails. This is good circumstancial evidence that the snails are near to normality and are not osmotically stressed. The extended head-foot is, of course, moist and the surface mucus has a high water content, but the possibility of the snails involuntarily taking in vast amounts of water does not exist. The only observable drawback of the method - apart from the effects of carbon dioxide which are discussed below - is that it appears to encourage mucus production. Wilson (1968b) has described the complex chemical nature of the mucus of Lymnaea truncatula, and Burton (1965) has suggested that haemolymph plays a large part in mucus production. The possibility therefore exists that mucus production during anaesthetisation causes depletion of some of the constituents of haemolymph. However, mucus production during the early period of anaesthesia, used for haemolymph collection, does not appear to be excessive, and the rapid recovery of snails is again reassuring.

It appears that carbon dioxide produces the deeper anaesthesia, but its acidic nature will affect haemolymph and thus it cannot be used in the collection of haemolymph for the determination of e.g. Ca^{++} and HCO_3^-. The effects of carbon dioxide on the pH of haemolymph

are described in Part III. The ionic changes produced by diethyl ether are described by Huf (1934) who also reviews the earlier literature. These and the effects of mucus production are really impossible to check. Accepting that anaesthetisation is a necessary evil and that the use of nitrogen provides an alternative to carbon dioxide when required, the gaseous methods developed here do allow adequate access to haemocoele for haemolymph collection.

A further factor deserving consideration is that the haemolymph of terrestrial snails is anyway extremely variable in composition. Many authors working on haemolymph composition, and on the effect of the environment on the snail, have recorded this (e.g. Duval, 1930; Kamada, 1933; Lustig Ernst and Renn, ~~et al~~, 1937; Meyer and Thibaudet, 1937; Martin, 1961; Roach, 1963; Robertson, 1964; Trams et al, 1965). Although there is little information of the effects of this variability on larval trematode parasites, Kendall and Ollerenshaw (1963) show that Fasciola hepatica in Lymnaea truncatula tolerates a wide range of environmental changes, and will survive starvation or aestivation of the host. Thus a small dilution of haemolymph, or a small loss of ions due to mucus production, assumes less importance and the possible effects of anaesthetisation on haemolymph appear less frightening. It can be reasonably expected that, using well-nourished snails, an accurate analysis of haemolymph collected using the rapid gaseous anaesthetisatio

methods should define an environment well within the range in which larval Fasciola hepatica will develop. This, and the rapid recovery of snails from anaesthesia were considered to be sufficient reasons for proceeding with analytical work on haemolymph collected using gaseous anaesthetisation.

Figure 1. Apparatus used in the collection of haemolymph from haemocoele.

1. Plasticine block.

2. Filter paper strip.

3. Micropipette.

4. Point of insertion.

5. Rim of shell is forced down to swell anterior haemocoele.

6. Attachment to micro-manipulator (diagrammatic).

7. Mouth-tube.

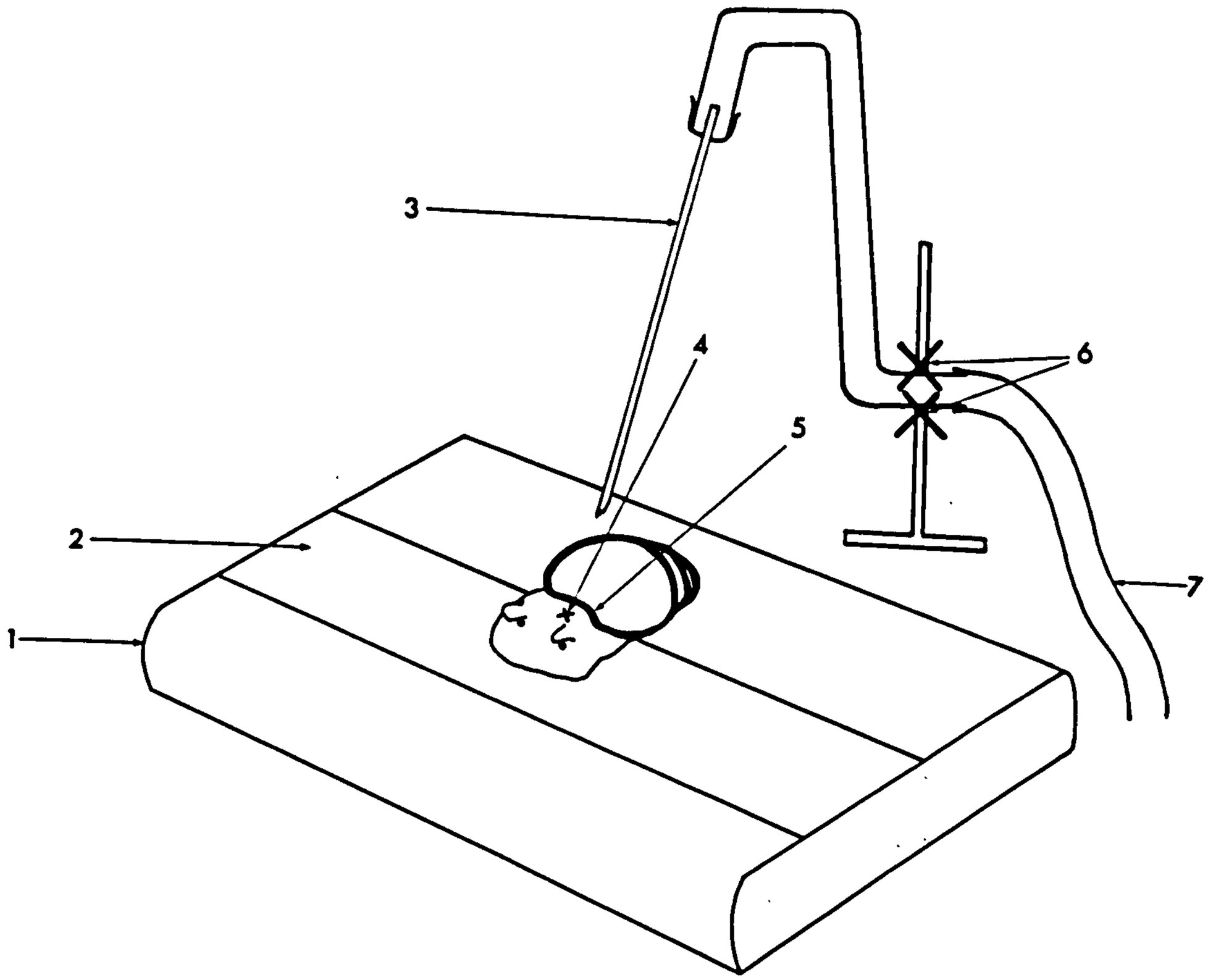

FIGURE 1

Plate 4

A snail held in the extended position with the rim of the shell forced down to swell the anterior haemocoele. The adjacent storage tube contains haemolymph and is capped with parafilm.

PLATE 4

PART III The composition of haemolymph

Introduction

The limiting factor in an analysis of the haemolymph of Lymnaea truncatula is the availability of material. With the method of collection described, 20 to 25 snails are required for each 20µl pooled sample. Such a sample is adequate for most chemical determinations, but does not allow analysis of some of the trace components of haemolymph. Thus no attempts are made to determine trace cations such as iron and copper or trace organic substances such as vitamins. The rather incomplete analysis described here took nearly 2 years to achieve, largely because of the developmental work required for the micro-analytical techniques, but also because a very large population of snails had to be maintained to allow sufficient repeats of determinations. The analytical methods adopted are all noted for their extreme sensitivity.

Quantitative determinations of Na^+, K^+, Ca^{++}, Mg^{++}, Cl^-, HCO_3^- and PO_4^{---} in haemolymph are described, and the errors involved discussed in each case. Work on the organic constituents of haemolymph was restricted, as the aim of the analysis was to supply information for an artificial haemolymph of a fully defined nature. Many organic constituents cannot be adequately defined or obtained in quantity as pure preparations. Thus work on protein is limited to a quantitative

determination of total protein and a brief attempt at electrophoretic separation. Similarly the work on lipid components is mainly qualitative. Quantitative determinations are given for most of the free amino-acids in haemolymph, and other trace amino-acids are identified. Glucose is shown to be the only soluble carbohydrate, and determinations of total nitrogen and non-protein nitrogen provide additional useful information.

In addition to the chemical determinations, the pH and depression of freezing point of haemolymph are determined, and a discussion compares Lymnaea truncatula with other species. Important raw data from the quantitative determinations are given in Appendix III.

Methods and Results

Each determination is considered separately in view of the length of the descriptions of some analytical methods.

(1) The measurement of the pH of haemolymph.

Methods

The pH of haemolymph was measured directly using the Radiometer pH meter PHM 25 and electrodes G252C and K150. The minimum sample volume required for these electrodes is 2µl. The pH of haemolymph, collected using both carbon dioxide and nitrogen during anaesthetisation, was determined. The electrode G252C was clamped vertically and the reference electrode K150 mounted on a micro-manipulator. Haemolymph samples were pooled in a 5µl capillary micropipette and the pooled sample read immediately after collection. Additional work pooling haemolymph in storage tubes allowed a comparison of the pH of centrifuged and uncentrifuged haemolymph. Ten determinations were made on haemolymph collected using carbon dioxide during anaesthetisation, and ten on haemolymph collected using nitrogen. Appendix III, Table 1 lists the measurements.

Results

The pH of haemolymph collected using carbon dioxide was determined as 7.53 ± 0.1, and that collected using nitrogen determined as 8.02 ± 0.1. The difference between these means is highly significant

(Student's 't' = 11.208, giving $p < 0.001$). Centrifuging has no measurable effect on the pH of haemolymph. The results show that the use of carbon dioxide as an anaesthetic could cause large errors in some determinations, although some authors (e.g. Roach, 1963: Trams et al, 1965) suggest that a variation of up to 0.3 pH can occur between individual snails anyway. In the present work, however, the observed difference is all the more significant because the samples were pooled. The pH of haemolymph collected using nitrogen is similar to that of other pulmonate haemolymphs (see Discussion), being well on the alkaline side of neutrality. Lockwood (1961) records a pH of 8.4 for Helix. The difficulty is making up physiological salines of high pH (Roach, 1963) is discussed in Part IV.

(2) The measurement of the osmotic pressure of haemolymph

Methods

The osmotic pressure of biological fluids is most conveniently determined by indirect methods such as the measurement of vapour pressure, depression of freezing-point, or conductance. Krogh (1939) summarises the early methods used, all requiring large amounts of sample, but Halket (1913) describes a vapour pressure method which could be scaled down for use with very small volumes.

The principle of this method is to set up droplets of sample fluid adjacent to droplets of standard salines in sealed capillary tubes of uniform bore. Each sample droplet is separated from the standard saline droplets bordering it, by air bubbles. Figure 2.b) shows a typical arrangement with only one sample droplet per tube. The lengths of sample and standard droplets are measured immediately after sealing the tube, and sets of tubes containing a range of standard salines are then kept at constant temperature measuring the lengths of the droplets until equilibrium is reached. Percentage changes in droplet length are then plotted against the concentration of the standard solutions. This gives a V-shaped curve to which the standard solution concentration axis is a tangent, at the point where no change in droplet length would occur i.e. the solution which would have the same vapour pressure, and hence the same osmotic pressure, as the sample.

This method was tried using 2 mm bore melting-point tubes (Electrothermal Ltd.) and 20, 10, 5, and 1 μl capacity Shandon microcapillaries both with and without a silicone treatment ("Repelcote", Hopkins and Williams). The droplets were positioned in the tubes by a screw-controlled suction device, similar to that used in manometric apparatus. The tubes were sealed using a micro-bunsen flame, or plugged with a thick silicone grease (Edwards High Vacuum), and held in rows on glass slides by rubber bands. Tubes were incubated at 25°C for 7 days, measuring droplet lengths daily with a travelling microscope. Many trial determinations were made, using test salines. Figure 2.a) and Appendix III, Table 2.a, show a typical result using 20 μl siliconised microcapillaries as tubes, and a test saline made up between 0.4 and 0.6% NaCl. No determinations were made on haemolymph using this method.

Freezing-point methods have been widely used in osmotic pressure determinations but are often beset with errors. Drucker and Schreiner (1913) were first to eliminate errors due to super-cooling, suggesting that in fact determinations should be made on the melting-point of a ready-frozen sample. Fritsche (1916) applied this principle in measurement on Daphnia blood and Ramsay (1949) developed from it a method requiring only 0.1 μl sample volume. The method has been

further elaborated by Jones (1941) and Gross (1954), with the introduction of polarised light to aid the viewing of frozen crystals. Jones (1941) used the method for 10 μl samples, employing a large range of standard solutions and direct comparison of melting-points, thus not needing a thermometer. Gross (1954) used only 3 standard solutions of known melting-points and used a kymograph to calibrate his apparatus, assuming its rise in temperature to be linear with time.

An apparatus was constructed similar to that used by Gross (1954) (see Figure 3., Plates 5 and 6), but using direct temperature measurements with a Beckman thermometer. The apparatus was contained in a Jencons expanded-polystyrene container with holes cut in the base and lid for a light-tunnel, and for insertion of the thermometer and stirring device. One inch diameter polariod discs were inserted at the base of the light tunnel, and on the top of the lid below the viewing objective. This top polariod was freely rotatable to obtain 'crossing' of polarisation axes. The cooled bath was a glass 'Monax' crystallising dish containing 600 ml of 30% ethanol (Jones (1941) and Gross (1954) used brine). The bath was supported in the apparatus by a cylindrical expanded-polystyrene block, bored centrally to form the light-tunnel, and was further insulated with cotton-wool padding. The stirrer was a small polythene propeller set on a silver-steel shaft and driven by a "Minidrill" electric motor (see Part II). The motor was powered by a 9 volt dry battery and stirring speed controlled by a rheostat,

to minimise vibration of samples during viewing.

The sample tubes (Plate 6) were heat-sealed 1 or 5µl capacity Shandon microcapillaries secured to a microscope slide by rubber bands. During determinations, the slide rested on 2 solid watch-glasses and carried screw-clamps at each end. The extra mass provided by these helped to cut down vibration due to stirring currents. Sample tubes were filled as for the vapour pressure method above. A row of tubes were viewed simultaneously on the slide using a dissecting microscope with X18.5 magnification.

The alcohol bath and watch-glasses were cooled outside the apparatus by adding small pieces of solid carbon dioxide, or pouring in liquid nitrogen. When cooled to about -10°C, the bath was placed in the apparatus and the frozen samples on the slide carrier placed inside. The slide-carrier and sample tubes were always pre-frozen before introduction to the bath, to ensure complete freezing of samples (supercooling is otherwise a possibility) and to speed up attainment of thermal equilibrium. The temperature -10°C was chosen to ensure thermal equilibrium between samples and coolant well before the temperature rose to the critical range over which melting-points were measured.

In practise, the temperature of the bath rose by about $2^{o}C$ per hour. The heat-filtering glass probably had little effect. The light source was a spot-light taken from a Vickers dissecting microscope, fitted to a transformer to vary the intensity of illumination. Heat conduction down the thermometer and stirring shaft probably accounted for most of the rise of temperature. A rise of $2^{o}C$ per hour was perfectly acceptable for the determinations to be made.

Preliminary work showed that the main difficulty was to establish criteria for deciding when a sample had actually melted. This is mentioned by other authors (Jones, 1941; Ramsay, 1949; Gross, 1954). With the present apparatus, long sample droplets of volume 0.2 to 1.0 μl melted over a temperature range of as much as $0.2^{o}C$. To minimise this difficulty, only very small samples were used (0.1μl) and sets of tubes were arranged so that tubes for each determination contained samples of as near as possible equal volume. The melting-point of the sample was then considered to be the temperature at which the last ice crystal had just melted. This stage could be very precisely observed, as the difference between glowing crystals, however small, and the fully translucent melted sample, was very sharp in the polarised light. During the determinations, the polaroids were kept fully 'crossed'.

First determinations compared the suitability of 1 and 5µl microcapillaries as sample tubes. These were not treated with silicones. Tubes were prepared containing sample droplets of distilled-deionised water, 0.5% NaCl, 1.0% NaCl, and freshly collected centrifuged haemolymph. The latter was from a pooled sample, collected using nitrogen in the anaesthetisation process. Tubes were stored in the deep-freeze (-25°C) between determinations. Results are given in Table 2.b. (Appendix III) for 5 determinations, each slide carrying 2 tubes only: a haemolymph sample or NaCl standard, and a water tube. The haemolymph results are summarised below. In this and all determinations, the Beckman thermometer was read to 0.001°C using a hand lens.

Following this, 2 sets of determinations were made on haemolymph from 2 large pooled samples, prepared as above. Each set comprised 5 separate determinations on separate sample tubes, with a water tube and 0.25%, 0.5% and 1.0% NaCl standards included each time and using 5µl tubes throughout. Thus 10 runs were made using the apparatus, and each took approximately 6 hours. Table 2.c. (Appendix III) gives the results, and the results for haemolymph are summarised below (Table 5). The depression of freezing point for haemolymph was thus determined directly, and by plotting the depressions found for the NaCl standards, and NaCl solution having the same osmotic pressure as haemolymph was also determined.

Figure 2.

SEE PAGE 149

Figure 3.

SEE PAGE 150

Results

The vapour pressure method (Figure 2, Table 2.a. (Appendix III) was highly unsatisfactory. The point at which the curve touches the axis is obscure, and equally unsatisfactory plots were obtained using either mean or percentage charges. The accuracy of the method could probably be improved by using larger samples in finer bore tubes giving greater length charges. These, however, would take even longer to equilibrate, and the technique would become even more tedious and time-consuming. For the purposes of accurate determinations on haemolymph, the method was considered a failure, and at no time did it successfully determine a test saline to more than $\pm$ 0.05% NaCl accuracy.

The freezing-point method was, however, a success. Plate 6 shows a frozen sample viewed through crossed polaroids. This photograph was actually taken on the Zeiss photomicroscope, and in the apparatus proper, the crystals glow much brighter against a darker background than is depicted here. The magenta colouration is due to phase retardation by the perspex enclosing the polaroids and this effect was seen in the apparatus proper.

Preliminary determinations gave Δ haemolymph = $\underline{0.286^{\circ}C}$ in the 1μl sample tube and Δ haemolymph = $\underline{0.283^{\circ}C}$ in the 5μl sample tube. The 5μl tubes were much easier to work with and were thus used for

all other determinations. Table 5 gives the results of the 10 determined samples from 2 haemolymph pools (see Table 2c) (Appendix III) for full data).

Table 5 - Summary of Melting-point determinations

Sample	Δ (°C) i.e. Mean $\pm$ standard deviation
Haemolymph (Pool 1)	0.2799 $\pm$ 0.018
Haemolymph (Pool 2)	0.280 $\pm$ 0.046
0.25% NaCl	0.159 $\pm$ 0.030
0.5 % NaCl	0.299 $\pm$ 0.035
1.0 % NaCl	0.592 $\pm$ 0.082

Ramsay (1949) gives Δ 0.5% NaCl as between 0.295 and 0.305°C, and Δ 1.0% NaCl as between 0.595 and 0.605°C and the above results are in full agreement, showing good linearity.

By plotting Δ against % NaCl, it was calculated that haemolymph is osmotically equivalent to a solution of NaCl of 0.465% $\pm$ 0.027%.

(3) The determination of the total weight of solids in haemolymph

Methods

5 µl sample volumes of haemolymph were taken from freshly collected, centrifuged, separate pools. The samples were held in 5 µl Shandon microcapillaries of known weight. The anaesthetisation process during collection included the use of carbon dioxide. The microcapillaries containing the samples were placed on glass slides and dried at 105°C for 24 hours in a 'hot box' oven. After cooling in a dessicator they were weighed and the dry weights of samples calculated. All weighing was performed on the 0 to 1 mg scale of the Cahn gram-electrobalance, using an empty microcapillary as a counterweight. As a rough check on the accuracy of the method, the technique was repeated using 3 5 µl samples of Wellcome chemical control serum, of known solid content (76.6 mg/ml). The drying process undoubtedly destroyed the bicarbonate content of haemolymph, but the control serum contained no bicarbonate. Table 3 (Appendix III) gives the results for 10 haemolymph samples.

Results

The mean dry weight of 5 µl of haemolymph was found to be 94.0 µg (standard deviation, ± 9.0 µg). Thus haemolymph contains 18.8 mg/ml total solids ± 1.8 mg/ml.

The 3 dry weights recorded for the chemical control serum samples were 391.4, 391.2 and 389.4 µg, and the mean of these gives a total solid content of 78.14 mg/ml, which is very close to the known value, 76.60 mg/ml, showing that drying in microcapillaries is a sufficiently accurate method.

(4) The determination of sodium and potassium in haemolymph.

Methods

No simple colorimetric methods are available for the determination of alkali metals although Glick (1963) reviews those that have been developed. With such a small sample available, methods involving precipitation and filtration procedures are best avoided. In recent micro-analytical work, direct emission flame photometry is used as the simplest and most accurate method of determination (e.g. Roach, 1963; Trams et al, 1965; Burton, 1965; Wilson 1968b).

The only disadvantage of flame photometry in the analysis of biological fluids is that there is mutual interference between the ions Na^+, K^+, and Ca^{++} and also important errors occur due to the presence of phosphate and protein. A considerable time was spent in the present study trying to forecast such effects in haemolymph analysis, by determinations on Wellcome chemical control serum, and on solutions containing known amounts of interfering ions. This work, and the other relevant literature, reviewed by Dean (1960) showed that interference effects in haemolymph analysis could be ignored with one exception: the enhancement of potassium readings by interfering sodium. The low levels of protein, phosphate and calcium and potassium in haemolymph samples for flame photometry have negligible interference effects.

The high level of sodium in haemolymph does interfere markedly in potassium estimations. Many authors of previous work do not even mention this, but Roach (1963) does eliminate mutual interference effects between Na^+, K^+ and Ca^{++}. The instrument used in the present study - an EEL. flame photometer supplied with Na, K, and Ca filters, and fitted with a rapid sampling attachment - was not sensitive enough for the determination of calcium in haemolymph. Brief work showed that its sensitivity for sodium and potassium determinations could be greatly increased by the use of organic solvents in samples and calibration solutions (Dean, 1960). The sensitivity for sodium was approximately doubled by using 60;40 isopropanol/water as solvent. Simple aqueous solutions were, however, adequate for the sodium and potassium determinations, and the work with organic solvents was not pursued, as an atomic absorption flame photometer became available for calcium determinations.

During calibration and haemolymph determinations, samples were atomised from 10 ml glass beakers in preference to the EEL cells or the rapid sampling attachment. A calibration curve was constructed for each set of determinations. The minimum volume of fluid required for a reliable reading was 1 ml, but normally 2 readings could be made from a sample of this volume

when the instrument was fully stable. Calibration curves were constructed for sodium using 10 solutions from 5 to 50 ppm Na^+ concentrations, and for potassium using solutions from 1 to 10 ppm K^+ concentrations. Distilled - deionised water was used to set the zero for sodium determinations. For potassium determinations, the zero solutions and all standards were made up to contain 17 ppm Na^+ to eliminate interference effects. Typical calibration curves are given in Table 4 (Appendix III), and Figures 4.a) and 4.b). Calibration stock solutions were made up using dried Analar NaCl and KCl.

As a check on the accuracy of the instrument, 5 determinations were made on chemical control serum, known to contain 128 mEq/litre Na^+. Samples were prepared by diluting 10 μl. volumes of serum to 1 ml final volume, and interference effects were ignored.

Haemolymph was collected using carbon dioxide in the anaesthetisation process, and samples prepared as for the control serum 10 samples were prepared for sodium from freshly collected, centrifuged, unpooled haemolymph. These were read with the instrument set for 50 ppm Na^+ full-scale deflection. For potassium, 15 μl haemolymph samples were diluted to 1 ml final volume and 10 such samples read, with full-scale deflection set for 10 ppm K^+. During the determinations, the zero-point, full scale deflection and response of the instrument were frequently checked by spraying calibration solutions, and distilled-

deionised water - containing 17 ppm Na^+ in the potassium determinations - was sprayed between haemolymph readings. The sodium and potassium present in haemolymph were calculated from the calibration curves.

Results

Full calibration data and readings for haemolymph are given in Table 4 (Appendix III). The instrument remained steady, throughout determinations and reading to the nearest 0.5 scale units was easily possible. The 5 determinations on chemical control serum gave deflections from 78.5 to 82.5 scale units, with mean deflection 80.4. From the calibration curve for sodium, this represents 30.1 ppm Na^+, i.e. a concentration of 3010 ppm in the original sample. This is a concentration of 130.9 mEq/litre of Na^+, which is very near the known value of 128 mEq/litre.

The concentration of Na^+ in haemolymph was determined as $\underline{1126 \pm 109 \text{ ppm}}$, that is $\underline{49.0 \pm 4.7 \text{ mEq/litre}}$. The concentration of K^+ in haemolymph was determined as $\underline{94.7 \pm 11.5 \text{ ppm}}$, that is $\underline{2.42 \pm 0.3 \text{ mEq/litre}}$.

(5) The determination of calcium and magnesium in haemolymph

Methods

Carbon dioxide cannot be used in anaesthetisation during the collection of haemolymph for calcium and magnesium determinations. The softening of the shell by the acid gas almost certainly indicates the dissolution of inorganic shell material and a consequent rise in the levels of both cations in the haemolymph. Thus nitrogen was used in anaesthetisation prior to all the accurate determinations described here. The use of nitrogen, as mentioned in Part II, does not produce a very deep anaesthesia and makes it difficult to collect large volumes of haemolymph. For this reason, fewer repeats of determinations were possible than in the case of sodium and potassium.

Atomic absorption flame photometry was selected as the simplest and most accurate method of determination. Methods, errors, and interference effects are discussed by Willis (1960a, 1960b, 1961), Dean (1960), and Zettner and Seligson (1964). The instrument used was an EEL atomic absorption flame photometer, and the method sheets supplied discount interference effects from Na^+, K^+ and Mg^{++} in calcium determinations. Willis (1960a) estimates that typical mammalian serum levels of Na^+ and K^+ cause a 1 to 2% over-reading error when calcium estimations are made on serum diluted 1:10. In the present study, haemolymph samples were diluted 1:50, and,

with the lower levels of Na^+ and K^+ in haemolymph, the above interference effect becomes negligible. Phosphate interference is, however, very important, and only a small amount is needed for reading to be significantly depressed (Wilkis 1960a, 1960b, 1961; Dean 1960). The EEL method sheets quote the following example for a 2.0 ppm solution of Mg^{++}, prepared as $MgCl_2$:

1) For the pure solution, deflection = 5.5.

2) With 2 ppm PO_4^{---} present, deflection = 4.9

3) With 10 ppm PO_4^{---} present, deflection = 4.4

The phosphate content of Lymnaea truncatula haemolymph is very low (see Part III 7.)) and would give a concentration of not more than 0.1 ppm in samples as diluted here for atomic absorption work. This would produce a 1 to 2% depression of readings at the very worst, and thus no attempts were made to correct for phosphate interference.

The effects of protein are less predictable. Willis (1960a, 1960b) suggests that interference from protein varies with the composition of the flame used, and with the height in the flame at which absorption is measured, and also mentions that the effects of protein and phosphate largely cancel each other out. The concentration of protein in haemolymph diluted 1:50 for atomic absorption

work is very low (see Part III 11.)) and far below the levels quoted by Willis (1960a, 1960b). Thus it appeared permissible to ignore the effects of protein interference and to simply dilute fresh haemolymph for determinations.

Roach (1963) showed that about 20% of the total calcium in haemolymph could exist "bound" to protein. For the purposes of the present analysis, the levels of free Ca^{++} and Mg^{++} in haemolymph are the more useful data, and only a brief determination was made on de-proteinised haemolymph to estimate the "bound" fraction of calcium. Roach (1963) gives no comparable data for magnesium, prefering a colorimetric estimation in which protein precipitation is automatic.

For calibration, calcium stock solutions were made up by dissolving $CaCO_3$ (Analar) in a minimal quantity of analytical grade HCl, and diluting to volume. Magnesium stock solutions were made up by direct dissolution of $MgSO_4 \cdot 7H_2O$ (Analar) in distilled-deionised water containing Cl^- ions equivalent to the Mg^{++} present. This addition of Cl^- was to correct a slight inaccuracy found when magnesium is sprayed as the sulphate. The EEL method sheets quote the following example, for a 2.0 ppm solution of Mg^{++}.

1) Sprayed as $MgSO_4 \cdot 7H_2O$, deflection = 5.2

2) Sprayed as $MgCl_2 \cdot 6H_2O$, deflection = 5.5

3) Sprayed as $Mg(NO_3)_2 \cdot 6H_2O$, deflection = 5.5

The 5% under-reading is corrected if equivalent Cl^- is added. Thus appropriate amounts of analytical grade HCl were added to the magnesium calibration solutions. This was not necessary for haemolymph samples where Mg^{++} is balanced almost entirely by Cl^- and HCO_3^-.

The air/acetylene flame was used for all determinations. This gives a slightly non-linear response with magnesium but provides much greater sensitivity than the alternative nitrous oxide flame. A preliminary calibration curve for calcium was constructed using solutions from 0 to 10 ppm Ca^{++}. Following this a very old sample of haemolymph of 100 μl volume was diluted to 5 ml final volume, and sprayed to determine the approximate level of free calcium in haemolymph. This large pooled sample had been collected using carbon dioxide during anaesthetisation and had been stored at - 25°C for 3 weeks.

Further preliminary calcium determinations were made using deproteinised samples. Here the blank and calibration solutions were made up to contain 4% trichloroacetic acid and 2500 ppm strontium ions. The strontium ions eliminated the possibility of increased phosphate interference after deproteinisation. Following calibration 2 haemolymph samples were prepared from 40 μl pooled

lots, collected using carbon dioxide during anaesthetisation. Each 40 µl sample was diluted to a final volume of 2 ml, adding first 1 ml of 8% aqueous trichloroacetic acid, and then 0.5 ml of 1.8102% aqueous strontium chloride, and finally 0.46 ml of distilled-deionised water. The samples were centrifuged at approx. 4,000 r.p.m. for 10 minutes, and the supernatants transferred to EEL cells for spraying. Willis (1960a) quotes 2.5 ml as the minimum volume required for a reliable reading, but it was found here that 2 ml was just enough if the cells were tilted during spraying. Table 5. (Appendix III) gives the calibration data.

Following this preliminary work with the unreliable calcium samples affected by carbon dioxide, 6 haemolymph samples were prepared using 20 µl pooled lots, collected using nitrogen during anaesthetisation. The instrument was re-calibrated twice during these determinations and Table 5.(Appendix III) summarises the data. The haemolymph samples were sprayed after direct dissolution with distilled-deionised water to 2 ml final volume. For all calcium determinations described above, the instrument was set as follows 1) Current setting = 3

2) Air pressure ≃ 10 psi. 3) Acetylene pressure = 8 psi

4) Slit width = 0.05 mm. 5) Wavelength = 423 mµ .

For magnesium, an initial calibration of 0 to 3 ppm Mg^{++}

showed the non-linear response (Figure 4.d)). A single 10 µl sample of haemolymph, collected using carbon dioxide during anaesthetisation, was diluted to 2 ml final volume and sprayed to determine the approximate level of magnesium in haemolymph. As for calcium, this was an old sample which had been stored at -25°C for 3 weeks. The results of this preliminary determination (see below and Table 5. (Appendix III)) showed that a calibration from 0 to 2 ppm Mg^+ would be more suitable. Following this, 6 haemolymph samples were collected, prepared and sprayed as described for calcium above. Table 5. (Appendix III) gives the data. For all magnesium determinations, the instrument was set as follows 1) Current setting = 1. 2) Air pressure = 10 psi. 3) Acetylene pressure = 8 psi. 4) Slit width = 0.1 mm. 5) Wavelength = 285.2 mµ.

Results

The preliminary determination on the old haemolymph samples, gave levels of calcium in haemolymph of 197.5 ppm (9.88 mEq/litre) for free calcium and 272.5 ppm (13.63 mEq/litre) for free + "bound" calcium in the deproteinised samples. This result suggested a level of free calcium in haemolymph.of between 100 and 200 ppm, and showed that about 30% of the total haemolymph calcium can exist in the "bound" state.

From the 6 accurate determinations (Table 5. (Appendix III)) it was calculated that the calcium level in haemolymph lies between 132 and 195 ppm i.e. between 6.6 and 9.8 mEq/litre. There is such a wide variation in the results, that further calculation of a mean $\pm$ standard deviation are worthless.

The preliminary determinations for magnesium gave a level of magnesium in haemolymph of 56 ppm (4.61 mEq/litre). Here the observed deflection was only 0.40 scale units, showing that a calibration from 0 to 2 ppm Mg^{++} was needed. The 6 accurate determinations (Table 5. (Appendix III)) give a mean level of magnesium in haemolymph of 50 ppm $\pm$ 1 ppm i.e. 4.20 mEq/litre.

Figure 4

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(6) The determination of chloride in haemolymph

Methods

A titrimetric method was chosen for chloride determinations, as a very accurate micro-burette was available. The instrument used was part of the Beckman ultra-microanalytical system, Model 150. Initial titrations using silver nitrate, with potassium dichromate as indicator, gave very vague end-points and this method was abandoned in favour of the method of Schales and Schales (1941).

Knights et al (1957) describe how the method of Schales and Schales (1941) can be scaled down for ultra-micro clinical work, and their modifications of the method were followed here. Reagents were pipetted from Beckman Sanz micropipettes from 10 to 140 μl capacity. Reagents used were as follows:

1) A working chloride standard, made up from dried Analar NaCl, containing 50 mEq/litre of Cl^-.

2) 0.03N HNO_2, made up from analytical reagent.

3) Indicator solution: a 100 mgm% solution of S-diphenyl carbazone (Eastman Kodak) in 95% Analar Ethanol.

4) Mercuric nitrate solution, made up by dissolving the Analar salt in about 50 ml distilled-deionised water, containing 1 ml of conc. HNO_3, to give a solution approximately 0.1N or 0.05N.

The 0.1N solution was used for initial trials using test salines and Wellcome chemical control serum, and the 0.05N solution for more accurate

work, and haemolymph determinations.

The titration procedure was the same for all determinations. 20 μl of sample, blank, and standard Cl^- solution, were pipetted to a small porcelain crucible and 160 μl each of 0.03N HNO_3 and the indicator solution were added. The mercuric nitrate solution was then run in from the micro-burette, stirring using the fitted vibration device, and titrating to the faint blue-violet end-point.

Four sets of titrations were performed. 1) 5 determinations on chemical control serum (certified as containing 91.5 mEq/litre Cl^-), accompanied by 5 blank titrations, and 5 titrations with a 50 mEq/litre Cl^- standard. 2) 5 determinations on a test saline known to contain 3.1327 gm NaCl/litre, accompanied by similar blank and standard titrations. 3) 3 titrations on separate haemolymph samples which had been stored at -25°C for 2 weeks. 4) 5 determinations on 20 μl samples of freshly collected, centrifuged haemolymph. All haemolymph was collected using carbon dioxide during anaesthetisation, and the old samples merely used to determine an approximate level of Cl^- before the 5 accurate determinations. Table 6. (Appendix III) gives the titration results.

Results

For the determinations on Wellcome chemical control serum, the mercuric nitrate solution was found to be 0.9659N and the serum

found to contain 96.99 mEq/litre chloride ions, which is very near the certified level of 91.5 mEq/litre. The data for these determinations (see Table 6.a. (Appendix III)) were rather suspect for 2 reasons. First, the burette was leaking during the blank titrations. Second, the end-points were all unintentionally 'cooked' to some extent, giving a false impression of fantastic repeatability of titrations. It was, difficult to avoid this with the burette dial visible, as the micrometer attachment controls the needle very accurately. Thus the burette can be adjusted and read to 0.001 µl accuracy around the end-point. To prevent this, the burette dial was kept covered during all further determinations and the end-point judged solely by eye. The porcelain crucibles used as titration vessels proved highly satisfactory, and were better than the Beckman vessels supplied, particularly for showing colour changes. The titrations were very rapid, and end-points sharply defined. Some precipitation was observed in control serum and haemolymph titrations. This was probably due to protein, and did not hamper the titrations in any way. Precipitation is not mentioned by Knights et al (1957).

The determinations on the test saline proved again the accuracy of the method. Here the mercuric nitrate solution was found to be 0.04851 N, and the saline found to contain 3.1424 gm NaCl/litre, which is near the known level of 3.1327 gm/litre. In terms of actual amounts measured, the 20 µl samples contained 62.65 µg NaCl, and were determined as containing 62.85 µg. The error here is about 0.3%

and represents 0.12 µg of measured Cl^-. The agreement between titration readings (Table 6.b. (Appendix III)) is good, and more realistic than the readings for the serum titrations above.

For the haemolymph determinations, the mercuric nitrate solution was again 0.04851 N, and the 3 determinations on odd samples gave a level of Cl^- in haemolymph of 31.5 mEq/litre. For the 5 determinations on fresh haemolymph, the lowest titration value gave a Cl^- level of 31.99 mEq/litre, and the highest a level of 32.36 mEq/litre. The mean determined level of Cl^- in haemolymph was determined as 32.14 mEq/litre $\pm$ 0.33 mEq/litre, i.e. 1410 $\pm$ 12 ppm.

(7) The determination of bicarbonate in haemolymph

Methods

Bicarbonate is perhaps the most difficult anion to determine in biological fluids. The equilibria between gaseous CO_2, carbonic acid, bicarbonate, and carbonate ions present a complicated picture. Added to this, the presence of solid $CaCO_3$ in mollusc tissues, and the buffering effects of proteins, complicates the issue even further. The aims of the present analysis must again be stressed here. Even if it were possible to determine all the above components and to describe the system as found 'in vivo', it would be impossible to reconstruct the system exactly in an artificial medium.

Robertson (1964) reviews work on ionic levels in haemolymph, and Wilbur (1964) has discussed the extensive literature on calcium metabolism in molluscs. All the work so far suggests that pulmonate haemolymph has a high pH, high "total CO_2 -content", and very low phosphate and sulphate content. The term "total CO_2 -content" means the total amount of CO_2 in all the components in the equilibria mentioned above. Hereafter, this is termed "total carbonate". From the previous work, the concept arises that the cations in haemolymph are almost entirely balanced by Cl^- and HCO_3^-. Thus Trams et al (1965) measured CO_2 displaced by acidification of samples and assumed that it was all derived from HCO_3^-. Similarly, Roach (1963) made determinations assuming that all the titratable alkalinity in

haemolymph was due to HCO_3^-, after making a small correction for phosphate. These are reasonable assumptions, and provide acceptable methods for approximately determining HCO_3^-, particularly as no colorimetric methods are available. Potts (1954) calculated the fractions of total carbonate existing as CO_2 -- lamellibranch haemolymph, and his results show that determination of HCO_3^- as total carbonate gives a very good approximation.

The present work followed the approach of Trams et al (1965) and other work directed towards the production of physiological salines. Thus the total carbonate content of haemolymph was determined by acidification, and measurement of the CO_2 evolved. Assuming that this was all derived from HCO_3^-, then the HCO_3^- required for an artificial haemolymph was calculated. Nitrogen was used exclusively in anaesthetisation procedure during haemolymph collection. Also it was realised that haemolymph starts to attain equilibrium with atmospheric CO_2 as soon as it is removed from the snail and hence haemolymph samples were used immediately after collection, with a minimum of time spent pooling and centrifuging.

For measurement of evolved CO_2, manometric methods were first considered but were rejected on the grounds of the cost and time involved in making apparatus for such small samples. Also micro-diffusion methods were rejected because of the size of sample required. The method finally chosen was the conductimetric method originally described by Fenn (1928) and Von Lebebur (1929) and further developed

by Prop (1954) for use in microrespirometry.

The method of Prop (1954) is suggested by Glick (1961) as a possible analytical method. The principle of the method is that CO_2 is absorbed by a dilute barium hydroxide solution and the decrease in conductivity of the latter, as barium carbonate is precipitated, is proportional to the amount absorbed. Conductivity changes are measured using special micro-conductivity cells, and a conductance bridge. Prop (1954) gives details for the construction of cells and Figure 5 and Plate 7. show a cell used in the present study.

Several cells were constructed, but the final calibration and haemolymph determinations were made using only one cell. No attempts were made to determine cell constants. The platinum electrodes were of 28 SWG wire, washed with 1:1 nitric acid and then repeatedly with distilled-deionised water, before electrolysis to produce the platinum-black coating. The cell was filled with a 3% solution of chloroplatinic acid, containing about 25 mg lead acetate. The external leads from the cell electrodes were joined to make them into a common electrode for the purposes of the electrolysis, and a further piece of platinum wire was arranged to dip into the neck of the cell to form the other electrode. Using a 2 volt accumulator, rheostat, and milliammeter, electrolysis was performed at 0.2 milliamps. for

1 hour, and then continued at 0.5 milliamps. for a further hour, examining the electrodes at intervals with a hand lens. After electrolysis, the cell was washed out thoroughly with distilled-deionised water, with which the electrodes were also kept covered when the cell was not in use. The lid of the cell was sealed with a silicone grease on the ground glass top flange (Edwards High Vacuum "Stopcock" Grease), and a thin coating of the same grease applied to the upper inside walls of the cell chamber (Figure 5) to allow accurate and stable positioning of sample drops. Liquid silicone treatments, such as 'Repelcote' (Hopkins and Williams) frequently used for treating microcapillaries, were not used here, because of the danger of contaminating the electrodes. The cell when in use was clamped with its long axis horizontal, and the electrodes were connected via screened leads to a Pye 11700 conductance bridge.

Prop (1954) calibrated his cells using pure CO_2, released from a small vessel inside the cell, measuring the change in volume after absorption by means of a manometric device attached to the cell. The measured change in volume was correlated with the change in conductivity. To avoid such manometric devices, and also tp provide a more suitable controlled calibration for the present work, it was decided to calibrate the cell by acidification of bicarbonate standards and then to use the same procedure for haemolymph samples.

Early work showed the importance of temperature control, as a bench lamp, used to illuminate the cell duing pipetting procedures

caused wide fluctuations in conductivity readings by its heating effect. Unfortunately, no sophisticated means of temperature control were available, but records of ambient temperature were kept throughout determinations. A very rough temperature coefficient of conductivity for the cell, containing 10 μl of approximately 0.1 N $Ba(OH)_2$, was determined by heating the cell using the bench lamp, and having the bulb of a mercury-in-glass thermometer clamped adjacent to the cell. Table 7.a. (Appendix III) gives the results.

The level of HCO_3^- in haemolymph was expected to be around 10 to 20 mEq/litre i.e. 600 to 1200 ppm. Standard bicarbonate solutions were made up from Analar $NaHCO_3$ in distilled/deionised water, to contain 250, 500, 1000 and 1500 ppm HCO^-. A litre of each standard was made up, and the solutions stored in full, tightly stoppered glass reagent bottles at 4^oC. The $Ba(OH)_2$ solution was approximately 0.1N, made up by dissolving 8 gm $Ba(OH)_2 \cdot 8H_2O$ in 500 ml boiling distilled-deionised water; filtering rapidly; and storing as the standard solutions. For convenience, a small volume of the $Ba(OH)_2$ solution was kept in the reservoir of a 10 μl Sanz micropipette. This was stored in a dessicator containing soda-lime.

Before use, the electrodes and cell chamber were rinsed with a jet of distilled-deionised water, and then quickly dried by spraying with Analar acetone, and a jet of compressed air, taking care not to blow in excess grease from the lid seal. 10 μl of the $Ba(OH)_2$

solution were then pipetted to the base of the cell from the Sanz micropipette. This operation had to be performed quickly, but carefully, without touching the electrodes. Prop (1954) recommends accurate centering of the pipette in the cell to avoid both the electrodes and the cell walls: the latter to prevent creeping of liquid between the pipette stem and cell wall by capillary attraction. No such creeping occurs with polythene Sanz micropipettes and it was found that the best pipetting method was to slide the micropipette down the wall of the cell, almost to the base of the electrodes, and then to empty it rapidly. After withdrawal of the micropipette, the sample droplet was quickly introduced from a 5 μl silicone-treated microcapillary, and positioned as shown in Figure 5. The cell was then clamped with its long axis horizontal, sealed, and left for a 5 minute equilibration period. During this time, the base of the cell was inspected with a hand lens to check the electrode coating for damage, and to check for air bubbles in the $Ba(OH)_2$ solution, around the base of the electrodes. If either of these conditions was noticed, the cell was emptied, cleaned, and re-set, with a repeat of the electrolysis if required. Conductivity drift was observed during the equilibration period, and the final stable reading recorded as the zero point for the determination.

After the equilibration period, the lid of the cell was slid aside just enough for the introduction of a 1 μl capillary micropipette containing 1 N HCl. The acid was pipetted directly into the sample droplet and the cell lid closed as the pipette was rapidly withdrawn.

A stopcock was started simultaneously with the introduction of the acid and conductivity was then measured at 1, 2, 3 and 5 minutes, and thereafter at 5 minute intervals until equilibrium was reached. Three determinations were made on each of the HCO_3^- standard solutions, and 4 determinations made on haemolymph. The data, and the linear calibration obtained by calculating the mean changes in conductivity, are given in Table 7. (Appendix III). Figure 6 shows a typical set of time/conductivity curves.

Haemolymph samples, collected using nitrogen during anaesthetisation, were pooled directly in the 5 μl micropipette and were thus not centrifuged. Each 5 μl pooled sample was immediately introduced to the conductivity cell and the total time for collection, pooling and introduction of a sample never exceeded 5 minutes. It was assumed that this short time, and the very small surface area presented by the pooled sample, prevented equilibration of the sample with atmospheric CO_2. Collection under oil would have avoided this problem entirely, but would have introduced difficulties in the manipulation of lighly anaesthetised snails; complicated pooling of samples; and made the micropipettes during collection very difficult to control.

Results

The method was fully satisfactory even in the absence of sophisticated temperature control. The data in Table 7.a) (Appendix

III) suggests a temperature coefficient of conductivity of around + 0.01 (mho x 10^{-2})* per ^{o}C rise in temperature. However a further calculation of the coefficient was made by comparing the highest and lowest recorded ambient temperatures with the zero points recorded for determinations at those temperatures using the same Ba(OH) solution For 16.2 and 20.0^{o}C, the recorded zero points were 0.314 and 0.327 (mho x 10^{-2})* respectively, suggesting a coefficient of around + 0.005 (mho x 10^{-2})* per ^{o}C rise. The calibration data in Table 7.b.) (Appendix III) suggest that the method is most reliable for determining samples of HCO_3^- above 1000 ppm concentration. The drops in conductivity observed for the more dilute standards are very small, and with a temperature coefficient of +0.01 (mho x 10^{-2})* per ^{o}C rise, a determination could suffer a 20 to 30% error even within the changes in ambient temperature permitted here. At concentrations above 1000 ppm it seems that around 5% agreement between triplicate samples can be expected. The spread seen in the drops recorded for haemolymph is a little higher than this. It is possible that the evolution of ammonia from biological samples could be responsible for this.

From the data in Table 7.b) (Appendix III) a straight line calibration curve was constructed. From this, the drops in conductivity measured for the 4 haemolymph samples were found to represent concentrations of 1080, 1090, 1150, and 1160 ppm HCO_3^-. Thus the total

* See page 97

carbonate content of haemolymph was determined as being between 1080 and 1060 ppm HCO_3^-, the mean value being 1120 ppm i.e. 18.36 mEq/litre.

* These conductivity readings were made on the 10^{-2} scale on the instrument used. As no attempts were made to measure cell constants, they are not meant to be taken as absolute values in mhos, but simply as arbitrary units on the Pye 11700 conductance bridge. It should be noted that drying the cell and electrodes with acetone did not appear to damage the platinum black coating.

Figure 5.

SEE PAGE 152

Figure 6

SEE PAGE 153

8) The determination of phosphate in haemolymph

Methods

All available information suggests that pulmonate haemolymph has a very low phosphate content (e.g. Roach, 1963: Robertson, 1964: Trams et al, 1965). There are several very sensitive colorimetric methods for the determination of phosphate including an elaborate scheme for the determination of separate fractions (Lowry, ~~et al~~, 1954). The method chosen for the present work was that of Allen (1940), in which the element phosphorus is determined, rather than the PO_4^{---}.

For the determination of free phosphate, the method was scaled down 50 times, giving the following volumes for colour development: sample or standard solution, 20 µl; 60% Analar perchloric acid, 16 µl; fresh amidol reagent, 16 µl; 8.3% aqueous ammonium molybdate, 8 µl. Beckman Sanz micropipettes were used throughout, and the reactions were performed in 2 x $\frac{1}{4}$ inch glass test tubes. After mixing the reagents by tapping and partial inversion of the tubes, 0.14 ml distilled-deionised water was added from a blow-out pipette, to give a final volume of 0.2 ml.

The level of phosphate in haemolymph was expected to be less than 0.5 M/ml (Trams et al, 1965) i.e. less than 15 ppm of the element phosphorus. A stock solution of 1000 ppm phosphorus was made up by dissolving dried Analar potassium di-hydrogen orthophosphate in

distilled-deionised water. Successive dilutions of this in chloroform-saturated distilled-deionised water gave working standards containing 5, 10, 15, 20 and 50 ppm phosphorus. These were stored at 4^{o}C. Duplicate tubes were prepared for each standard and for blanks containing 20 µl distilled-deionised water + all other reagents. Colours were read after 1 hour on the Hilger-Watts Uvispek spectrophotometer at 620 mµ. The instrument settings were: meter sensitivity, 7; input sensitivity, x 1; slit width, 0.4 mm, and the cells used were 0.2 ml capacity, 1 cm path silica micro-cells. Table 8.a. (Appendix III) gives the linear calibration obtained.

Following this, 2 sets of determinations were performed on duplicate haemolymph samples. i.e. 4 separate 20 µl lots of haemolymph. Haemolymph was collected using carbon dioxide during anaesthetisation, and was centrifuged and used immediately after collection. All the haemolymph samples showed protein precipitation after the addition of perchloric acid. This always remained as a coagulated mass and was easily avoided during pipetting before colour reading. Table 8.b. (Appendix III) gives the readings for haemolymph determinations and for the standards run concurrently.

For the determination of total phosphate, the method was again scaled down 50 times, and the same reagents used. Here, the 20 µl

sample was introduced to the base of a micro-kjeldahl tube, and 0.1 ml 60% Analar perchloric acid were added. Two small pieces of broken sintered glass were added to prevent 'bumping' and the mixture was heated on a digestion rack (Gallenkamp). The rear heating element was first turned full on, keeping the front element at its lowest setting, for 8 minutes. This ensured evaporation of all the water present, and fuming commenced. The rear element was then switched off, and the front element turned to its highest setting so that the perchloric acid refluxed in the cool upper tube. All heating was stopped after 10 minutes total time and the tubes allowed to cool thoroughly. The above digestion times were determined by brief trials using Wellcome chemical control serum, in which the digests became colourless after 9 minutes.

Initially, a residual volume of about 80 μl of digest was expected and for the first calibration 80 μl of fresh amidol reagent and 40 μl ammonium molybdate solution were added to the tubes. This, however, gave insufficient volume for the first colour measurement, and duplicates had to be pooled. Table 8.c.) (Appendix III) gives the readings. Suspecting contamination and absorption of digest by the sintered glass pieces, the calibration was repeated using the same volumes, but with powdered sintered glass in the tubes and with the addition of 40 μl distilled-deionised water to each tube

after colour development, to give an adequate final volume. Colours were read as for free phosphate. Table 8.d.) (Appendix III) shows the good linearity obtained.

Haemolymph samples were collected and prepared as for the free phosphate determinations above. The first set of haemolymph determinations used 3 pooled 20 µl samples, the digestion time being 10 minutes for water evaporation and 4 minutes refluxing the perchloric acid. Colorimetric procedure was as for free phosphate above, running 3 standard solutions (5, 15 and 50 ppm phosphorus) concurrently for calculation purposes. A second set of determinations was performed again using 3 20 µl samples; adding 0.2 ml perchloric acid to each tube, and extending the digestion time to one hour i.e. 15 minutes water evaporation time and 45 minutes refluxing the perchloric acid. Here enough volume of digest remained for the addition of water after colour development to be omitted. Here only 2 standards (2.5 and 5.0 ppm phosphorus) were run concurrently as the approximate level of total phosphate in haemolymph was now known. Table 8.e.) (Appendix III) gives the readings for the 2 sets of haemolymph determinations.

Results

The data in table 8.) (Appendix III) shows that for free phosphate determinations, the standards run with the first haemolymph were far

too concentrated. However, the good linearity of the calibration and the more dilute standards run with the second set of determinations made up for this. From the data, haemolymph 1 contained 2.37 ppm free phosphorus, and haemolymph 2, 2.60 ppm free phosphorus. The actual amount of phosphous measured was about 0.05 µg, and the variation in optical densities (± 0.003) was equivalent to about ± 0.006 µg of measured phosphorus over all the determinations ie. approximately ± 0.3 ppm in haemolymph. Thus the method was extremely sensitive.

The level of free (inorganic) phosphorus in haemolymph was found to be between 2.4 and 2.6 ppm i.e. 7.35 to 7.97 ppm phosphate ions (0.232 to 0.251 mEq/litre).

Similarly with the total phosphate determinations, the very low level in haemolymph was not predicted beforehand, and the standards run with the first set of haemolymph determinations were far too concentrated (Table 8)(Appendix III)). Agreement between triplicates for the 2.5 ppm standard and for the haemolymph samples appears poor, but for the former it is only equivalent to ± 0.006 µgm measured phosphorus, as with the free phosphate determinations, From the data, the first set of haemolymph determinations give a total phosphorus level of 2.92 ppm and the second set a level of 3.70 ppm.

The level of total phosphorus in haemolymph was determined as 2.9 to 3.7 ppm phosphorus i.e. 8.9 to 11.3 ppm phosphate ions (0.280 to 0.357 mEq/litre).

The results are not altogether satisfactory, but lack of haemolymph prevented any further repeats. The determinations do show the very low level of phosphate in haemolymph.

9) A note on the determination of sulphate in haemolymph

Nearly all the information available points to very low levels of sulphate in pulmonate haemolymph. The majority of the published analyses (see Robertson, 1964) omit figures for sulphate because of this. Trams et al (1965) give a level of sulphate for (Cepaea nemoralis haemolymph of "less than 0.5 µMoles per ml" . Potts (1954) records a level of 0.76 mg-ions per litre for Anodonta cygnea and Roach (1963) records the highest known level of 2.21 m-Moles per litre for Arion ater, using a conductimetric method with 100 µl samples (Paulson, 1953). Florkin (1943) records a level of 0.31 mg-ions per litre for Planorbis corneus, probably the nearest relative to Lymnaea truncatula for which a haemolymph sulphate value is known.

Unfortunately the conductivity cells used in total carbonate determinations were not suitable for sulphate analysis. Preliminary attempts were made to determine haemolymph sulphate using the colorimetric methods of Bertolacini and Barney (1957) and Spencer (1960). A lot of time and material was wasted in showing that no measurable optical densities could be obtained with either method, scaled down for use with a 20 µl haemolymph sample.

Regretfully, attempts to determine haemolymph sulphate were abandoned. Addition of empirical amounts of sulphate to an artificial haemolymph is discussed in Part IV.

10) The determination of glucose in haemolymph

Methods

Carbohydrate metabolism in the mollusca is reviewed by Goddard and Martin (1966), showing that glucose is the main soluble carbohydrate found in haemolymph. The presence of galactogen in many species, however, suggests that galactose may also be present. Also Fairbairn (1958) demonstrated that the non-reducing di-saccharide trehalose is found in invertebrates from many phyla, including the mollusca. Other possible soluble carbohydrates in haemolymph are amino-sugars and hexuronic acids, although Trams et al (1965) found no uronic acids in Cepaea nemoralis. Thus a chromatographic analysis of Lymneae truncatula haemolymph was first undertaken to see whether glucose was the only carbohydrate present.

Paper chromatographic techniques were taken from Smith (1960). Preliminary runs using isopropanol/water as solvent (160 ml/40 ml) gave very poor separation of glucose/galactose and glucosamine/galactosamine standard marker solutions. It was also found that aniline phthalate will not locate trehalose. The solvent eventually chosen was ethyl acetate/pyridine/water

(120 ml/50 ml/40 ml). Whatman No. 1 paper was used and the chromatograms were run with descending solvent in Shandon glass tanks. Using 1 μl spots of glucose, galactose, glucosamine, galactosamine, and glucuronic acid marker solutions (Shandon, 0.01 Molar) and a 1 μl spot of 0.01M trehalose dihydrate, excellent separation was obtained with runs lasting from 7 to 7½ hours at 20°C. The location reagent used was silver nitrate (Smith, 1960), which locates trehalose adequately. Three runs were then performed, including all the above standards except galactosamine, and spotting 10 μl of freshly collected haemolymph with each run. Haemolymph was collected using carbon dioxide during the anaesthetisation process. An additional marker spot, containing 1 μl each of Shandon amino acid mixtures 41 and 42, was included on each paper. This was to control the possible location of haemolymph amino-acids by the rather unspecific silver nitrate reagent, and their misidentification as sugars. Plate 8 shows a typical chromatogram.

For the colorimetric measurement of glucose the method of Somogyi (1952) was used. This is based on the reaction described by Nelson (1944) and requires a protein-free sample. De-proteinisation by violent methods, such as strong acids or TCA, is not advisable for samples used for carbohydrate analysis. The de-proteinisation method chosen was that of Somogyi (1945) in which protein is

precipitated by mixing equivalent amounts of $Ba(OH)_2$ and $ZnSO_4$ in the sample. For accuracy this requires a fairly large sample, and thus the sample used here was a large pooled haemolymph sample of 100 μl volume. This was collected using carbon dioxide in the anaesthetisation process. De-proteinisation was by the addition of 200 μl each of a 0.3N $Ba(OH)_2$ solution and a 5% hydrated zinc sulphate solution, previously matched so that equal volumes of each were exactly equivalent (by titrations using phenol phthalein as indicator). After thorough centrifugation, the protein-free supernatant provided 4 x 100 μl aliquots for glucose determinations, each containing glucose equivalent to 20 μl of original haemolymph.

The standard solution included in the colorimetric work was a 0.01% glucose solution working standard, made up by dilution of a 1% stock solution in 0.25% benzoic acid (to act as preservative). The stock solution was stored at $4^{o}C$. A calibration curve was constructed, showing good linearity, and the data are not given here. Triplicate standard and blank tubes were prepared concurrent with the 4 haemolymph samples. Colour development was in 2 x $\frac{1}{4}$ inch glass test tubes, the solutions being mixed as in Table 9.) a.) (Appendix III) to give a final volume of 0.3 ml. The tubes were heated in a rack over a boiling water bath for 10 minutes. Colours were read undiluted in 0.2 ml capacity micro-cells on the Hilger-Watts Uvispek spectro-

photometer at 510 mμ. The instrument settings were: sensitivity, 7; input sensitivity, x 1; slit width, 0.075 mm; Table 9.) b.) (Appendix III) gives the results.

Results

Glucose was identified as the only soluble carbohydrate in haemolymph (Plate 8). Spotting the 10 μl. haemolymph spots on to the paper was very difficult even when done a fraction of a microlitre at a time, because the proteins and salts present dried on the paper making it non-absorbent. On Plate 8 it appears that some hexuronic acids may also be present, but the location of amino acids shows the unreliable nature of the streak from the haemolymph spot (referred to on Plate 8 as the 'serum spot').

From the colorimetric data, it was calculated haemolymph contains between 800 and 850 ppm glucose, the mean optical density representing a value of 814 ppm. The glucose content of haemolymph is obviously a very important consideration in the development of culture media and it is unfortunate that neither time nor materials were available to repeat determinations on snails kept under different environmental conditions.

11) Determination of the total nitrogen and non-protein nitrogen in haemolymph

Methods

These determinations were undertaken to provide a useful comparison with known levels in other biological fluids and with levels in media in current use for 'in vitro' culture work. Previous analytical work in general omits any nitrogen determinations, although Trams et al (1965) include a figure for the total amino-groups in the haemolymph of Cepaea nemoralis. In Florkin's (1966) review of nitrogen metabolism in the mollusca, actual nitrogen determinations are restricted to work on the kidneys and excreta (e.g. Jezewska, Gorzkowski, and Heller, 1963).

The method used was based on that of Jacobs (1960, 1962). This method employs acid digestion of samples in sealed ampoules, avoiding the hazards of open tube digestion and the use of catalysts. The method is extremely sensitive, and all reagents were made up with very pure distilled-deionised water. Reagents used were the purest available - even Analar sulphuric acid is not pure enough for this method and causes very high blanks. Analar sodium acetate was, however, found to be pure enough and re-crystallisation (Jacobs 1960, 1962) was not necessary. The following reagents were used:

1) Wellcome chemical control serum with certified levels of TN and NPN. Whole serum was diluted 1:9 with distilled-deionised water to give a stock solution. This was stored at $4^{o}C$ for a maximum of 2 days.

2) Concentrated sulphuric acid (B.D.H. micro-analytical reagent).

3) 2.0M citrate buffer (Jacobs, 1960, 1962), pH 5.0.

4) Ninhydrin stock solution i.e. 4% (Koch-light) in re-distilled, purified methyl cellosolve (Jacobs 1960, 1962).

5) Stannous chloride (solid research reagent).

6) Normal and 4N sodium hydroxide (carbonate-free) (Griffin and George).

7) 4M acetate buffer (Jacobs 1960, 1962) pH 5.5.

For total nitrogen determinations the haemolymph samples and standard solutions were pipetted directly into the base of 10 x 1 cm pyrex test tubes. The tubes were then dried over P_2O_5 in a vacuum dessicator for at least 12 hours. 0.25 ml concentrated sulphuric acid was then added to each tube, and the tubes converted to sealed ampoules by drawing out the neck in the oxy-coal gas glame. When cool, each ampoule was rotated and inverted so that the entire inner surface was wet with acid. The ampoules were then placed in

pencil-labelled 100 ml pyrex beakers, and heated for 30 minutes at 460 to 480°C in a muffle furnace. After cooling, the ampoules were opened in turn, and the contents of each carefully diluted with 1 to 2 ml distilled-deionised water. When the heat of dilution had dissipated, the contents were transferred by pasteur pipette to 25 ml graduated flasks. Each ampoule and 'lid', broken off during opening, were then washed three times with distilled-deionised water and the washings added to the flasks. A fresh pipette was used for each ampoule.

To each flask was added 2.5 ml of 2M citrate buffer and a pre-determined volume of 1N carbonate-free NaOH. This volume was determined by titrating the contents of 2 or more extra blank ampoules with the NaOH to a final pH of 5.0, using a pH meter. The flasks were then filled to the mark with distilled-deionised water.

A working solution of ninhydrin was prepared by mixing 50 ml of the minhydrin stock solution, 25 ml distilled-deionised water, and 25 ml 4M acetate buffer: finally adding 0.08 g solid stannous chloride and stirring thoroughly. This solution was made up fresh before every set of determinations. A 2 ml aliquot from each flask was added to 2 ml of the working ninhydrin solution in

standard 15 ml glass test tubes. These were capped with 'Oxoid' metal caps and heated in a boiling water bath for 30 minutes. When cool, colours were read undiluted at 570 mμ on the Hilger-Watts Uvispek spectrophotometer. For all determinations the instrument settings were: Sensitivity, 8; input sensitivity, x 5; slit width, 0.05 mm.

Two attempts were made to construct a calibration curve. Both included triplicate blank ampoules containing sulphuric acid only, and triplicate standards containing 10 to 50 μl of the diluted serum i.e. 11.84 to 59.20 μgm of nitrogen. Three extra blanks were included each time to determine added NaOH Table 10 a) and b) (Appendix III) gives the readings taken, and the readings for 6 ampoules containing 5 separate 5 μl centrifuged haemolymph samples. These were run concurrent with the second calibration attempt. The haemolymph was collected using carbon dioxide in the anaesthetisation process, and was used immediately after centrifugation. The diluted serum stock solution used for standards contained 1184 ppm total nitrogen.

For non-protein nitrogen determinations, ampoule digestion, colour development and colour reading were essentially the same. The only changes were in actual volumes used, and in the introduction of a deproteinisation procedure. The method of deproteinisation

chosen should not leave residues in the dried sample that would affect the digestion procedure or introduce nitrogenous impurities. There are objections therefore to using e.g. TCA or picric acid. The method of Somogyi (1945) was used, as in glucose determinations (Part III 10.)). Deproteinisation, digestion, and colour development were first tried using 10 and 20 μl <u>undiluted</u> Wellcome chemical control serum standards. Four sets of triplicate conical centrifuge tubes were set up, 2 sets containing 20 μl distilled-deionised water, and the others 20 μl serum standard and 10 μl serum standard + 10 μl distilled-deionised water. To each tube was added 0.3 ml distilled-deionised water followed by 40 μl each of the $Ba(OH)_2$ and $ZnSO_4$ solutions. The tubes were then shaken vigorously on the "whirlimixer," (Fisons). The tubes were centrifuged, and supernatants transferred to the ampoule tubes, washing precipitates three times by further centrifugation and adding the washings to the first supernatants. The ampoule tubes were then placed in an oven at 105°C until the samples were nearly dry, when they were transferred to the P_2O_5 vacuum dessicator. After digestion, 10 ml graduated flasks, 1 ml volumes of citrate buffer and 4N carbonate-free NaOH were used in colour development. Table 10 c.) (Appendix III) gives the readings taken for standards and for 5 ampoules containing 20 μl haemolymph prepared, de-proteinised and treated as above, running concurrent standards of 5 μl undiluted control serum. The standards used

in non-protein nitrogen determinations contained 780 and 450 ppm non-protein nitrogen.

Results

The haemolymph samples were found to contain from 13.11 to 14.37 µg total nitrogen, with a mean value of 13.46 µg (standard deviation for 6 determinations = ± 0.501 µg). The variation in optical densities for the haemolymph samples represents 0.1 µg nitrogen in 1.08 µg actually measured. The level of total nitrogen in haemolymph was determined as 13.5 µg per 5µl ± 0.5 µg i.e. 2692 ppm ± 100 ppm.

Non-protein nitrogen for the 5 haemolymph samples was determined as 2.64 to 3.06 µg, the mean value being 2.91 µg (standard deviation ± 0.165 µg). The actual weights of nitrogen measured for haemolymph samples were 0.528 to 0.612 µg and the total spread of readings (0.014) represents 0.08 µg of measured nitrogen. The level of non-protein nitrogen in haemolymph was determined as 2.91 µg per 20µl ± 0.165 µg i.e. 145.5 ppm ± 8.25 ppm.

The relationship (TN - NPN) x 6.25 = Total protein gives a forecast of the expected level of total protein in haemolymph. For Lymnaea truncatula (TN - NPN) x 6.25 = 15.86 mg/ml. For total protein determinations see Part III (13).

12) Determination of free amino acids in haemolymph

Methods

A review of molluscan nitrogen metabolism (Florkin, 1966) shows that most of the analytical work on amino acids has been on the marine species. However, there is some information on pulmonate haemolymph gained mainly by chromatographic analysis. Targett (1962a, 1962b) and Kerkut and Cottrell (1962) have identified a wide range of amino acids in pulmonate haemolymph, the latter workers including quantitative determinations for Helix aspersa. Kerkut and Cottrell (1962) also realised the variability of haemolymph (see Part II, Introduction) and determined amino acid levels for snails in different states of hydration and activity (Wells, 1944). Friedl (1961c) gives an analysis of free amino acids in the haemolymph of Lymnaea stagnalis, using both paper and column chromatography. More recently, free amino acids in the haemolymph of Australorbis glabratus have been determined by Dusanic and Lewert (1963) and Gilbertson, Etge, and Ogle, ~~et al~~ (1967); and in the mucus of Lymnaea truncatula (Wilson, 1968b) showing the value of using an auto-analyser in such work. All the information available suggests that the levels of free amino acids in haemolymph are very low. Friedl (1961c) draws attention to this fact and to the need for vast amounts of materials to determine all compounds

quantitatively. The use of an auto-analyser overcomes this problem to some extent but it was never expected that all the amino acids in haemolymph would be determined quantitatively.

Preliminary work using thin layer chromatography enjoyed little success. Using one - and two - way solvent systems with MN-Cellulose pulver 300 (Mackery Nagel and Co.), 1, 5 and 10 µl spots of haemolymph were run with Shandon marker mixtures. Location with ninhydrin reagent revealed the following components: a trace of basic amino acids; a Glycine + serine spot; a threonine + alanine spot; a methionine + valine spot; a trace of proline; a trace of tyrosine; and a spot probably containing leucine, isoleucine and phenylalanine. A rough estimate of the density of these spots suggested that around 200 µl of haemolymph would be needed for each determination on a typical auto-analyser.

This problem was partially solved by using a very sensitive micro-column system developed for the Technicon auto-analyser. The very small amounts of material and the experimental nature of the apparatus still caused difficulties, but without this virtually untried piece of apparatus no quantitative work would have been possible. The micro-column used (Technicon) operates on the principle originally described by Moore, Spackman, and Stein, ~~et al~~ (1958) and requires

only 0.01 μMoles of each acid to give an integrateable peak on the recorder. The micro-column was operated under high pressure and the first one set up was ruined by a hair-line fracture. With a satisfactory replacement, chromatograms were run, using a device that expanded the input signals to the recorder by x 4 or x 10.

To gain experience in handling the apparatus, a preliminary chromatogram was run on a standard amino acid mixture supplied by Technicon. This contained exactly 0.01 μMoles of each amino acid. The autoanalyser was programmed for a 12 hour run and the recorder expander set at x 10. Unfortunately the early recorded peaks for aspartic acid, threonine and serine, went 'off-scale' and the recorder expansion factor was reduced to x 4. This gave integrateable peaks for all amino acids from glutamic acid to phenylalanine inclusive. After the phenylalanine peak, the base line and future peaks rose so much due to ammonia contamination on the column that no integration was possible. A repeat run, with the recorder expander set at x 4 throughout, gave a much better chromatogram, but here again ammonia contamination ruined all chances of integrating the peaks from the basic amino acids. All attempts to reduce the base line rise due to ammonia contamination failed. These included the preparation and washing of fresh columns with different buffer systems and the keeping of the apparatus in a smoke-free room.

It was decided that ammonia contamination on the micro-column could not be solved in the time available and that it was better to press on with chromatograms of haemolymph, at least hoping to identify all the amino acids present and to determine the major components quantitatively with the exceptions of the basic amino acids. Table 11a) (Appendix III) gives the results of the second chromatogram of the Technicon standard mixture. This was used as calibration data as each integrated peak represents 0.01 μMoles of acid.

All haemolymph used was collected by the carbon dioxide method of anaesthetisation. The first 12-hour chromatogram used a sample of 40 μl pooled, centrifuged, haemolymph that had been freeze-dried and taken up in 1 ml of 0.1N hydrochloric acid containing 0.01 μMoles of norleucine. The freezd-drier used was an all glass Quickfit M.F.45 model coupled to an Edwards High Vacuum pump (Speedivac E.S.35). It was suggested, (Humpherson 1968, personal communication) that chemical deproteinisation of small samples was unnecessary, and only served to introduce errors. Experience showed that the small amounts of protein in samples remained as a slight discolouration at the top of the column. The top few centimetres of resin were thus periodically discarded. The whole 1 ml prepared sample was pipetted directly on to the column. Table 11.b.) (Appendix III) gives the results of this first run

and the difficulties encountered. A similar chromatogram was then run using 50 µl original haemolymph sample again including a 0.01 µM norleucine standard to estimate variation between runs. Table II.c.) (Appendix III) gives the results. A final chromatogram was then run again using 50 µl original haemolymph sample, but not including a norleucine standard. During this run the recorder expansion factor was changed at intervals, with adjustment of the base line, to try to boost small peaks seen on the other runs up to integrateable size. Also, the colorimeter output leads were temporarily interchanged to transfer the signal for proline to the most sensitive recorder input line (the uppermost trace on this recorder). Table 11.d.) (Appendix III) gives the results of these adjustments and lists the peaks lost when making them.

For calculation purposes, the original calibration data, Table 11.a.) (Appendix III), and further chromatograms of Technicon standards (Humpherson, 1968, personal communication) provided adequate data. The latter runs also included ∝- amino-n-butyric acid, confirming the identification of this on the haemolymph runs: Table 11.b.) and c.) (Appendix III). Table 6 (below) summarises the results and shows the incomplete nature of the analysis. Lack of material prevented any further repeats.

Results

At least one quantitative determination was made on each amino acid present in realistic quantities, and other trace components were satisfactorily identified. Integrated areas of less then 1.0 units were not considered reliable enough for further calculations. Changing the recorder expansion factor did produce a reliable peak for α-amino-n-butyric acid but many peaks were lost in this final chromatogram because of difficulties in base line adjustment after changing the expansion factor. With so few repeats of determinations it is meaningless to calculate standard deviations for means. However the areas calculated for the included nor-leucine 0.01 μM standards may be compared. These were 15.02 and 16.65 units. The difference between these (1.62) represents 0.001 μMoles and shows that the reproducibility of chromatograms is fairly good. Table 6. summarises the results of calculations.

Table 6. Summary of findings, giving determined values of free amino acids in haemolymph

Compound	No. of times identified	No. of times determined	Determined values (mgm/litre)	Mean value (mgm/litre)
Cysteic acid	3	3	1.982	1.927*
			2.115	
			1.685	
Hydroxy-l-proline	3	0	-	-
Aspartic acid	3	2	5.511	6.935
			8.359	
Threonine	3	1	18.445	18.445
Serine	3	1	24.215	24.215
Glutamic acid	3	2	25.298	23.874
			22.450	
Proline	3	3	78.012	65.112*
			66.678	
			50.645	
Glycine	3	3	9.073	9.577
			9.622	
			9.035	
Alanine	3	3	38.969	40.795
			38.085	
			45.332	
∝-amino-n-butyric acid	3	2	2.256	2.820*
			3.383	
Valine	3	2	24.463	26.137
			27.811	
Cystine	3	2	6.212	5.648*
			5.083	

Compound	No.of times identified	No. of times determined	Determined Values (mgm/litre)	Mean value (mgm/litre)
Methionine	3	2	4.947	6.328
			7.709	
Isoleucine	3	3	7.413	8.150
			7.228	
			9.808	
Leucine	3	3	8.988	11.028
			11.324	
			12.863	
Tyrosine	3	0	-	-
Phenyalanine	3	0	-	-
Ammonia	3	3	4.032	5.289
Ornithine	2	0	-	-
Lysine	2	0	-	-
Histidine	2	0	-	-

*(These values are rather unreliable because of the small peaks used for calculations. In particular, the calibration peaks for proline were very small and the determined values appear ridiculously high. The values determined for ammonia appear reliable, but all the ammonia may not have come from the sample. The later base line rise shows the high contamination of the column and buffers with ammonia.)

The results are incomplete but do provide much useful information that no other technique could have produced with such small samples. It

is interesting that arginine was not located in any chromatograms; even when it was included in a mixture of standards (Table 11.a.) (Appendix III)). Thus there is the possibility that arginine does occur free in haemolymph. The relevant literature (Florkin, 1966; Wilson, 1968) suggests that arginine may be present in significant amounts. Humpherson (1968, personal communication) showed after much experimentation that arginine appears, on chromatograms run on the micro-column, up to 12 hours after the other basic amino acids. Adjustment of buffers and of the salt content of the eluting solvent can correct this, but this often causes other complications, such as poor threonine/serine separation.

The absence of taurine is in line with previous work (Florkin, 1966), as this amino acid is restricted to marine species. Another notable abscentee is tryptophan, as this was found in Lymnaea stagnalis (Friedl, 1961c). The above results are compared with known levels in other species in the discussion and summary of analytical work at the end of Part III.

13) Determination of the total protein content of haemolymph, with a brief attempt at electrophoretic separation

Methods

For the purposes of the present study, the determination of total protein in haemolymph is only of use in suggesting amounts of alternative macromolecules that should be added to an artificial haemolymph. The preparation of pure protein fractions from Lymnaea truncatula, for use in large scale culture work, would be impossible, requiring vast numbers of snails. Closely related larger species could possibly be used, however, and an electrophoretic separation of haemolymph proteins could give a useful guide to the types of molecules required. Electrophoretic studies have been performed by Woods, ~~et al~~ Poulsen, Engle, and Peet, (1958), Cheng, and Saunders (1962), and Wright and Ross (1963), and Cheng (1964) has pointed out the taxonomic value of such work. The only work on Lymnaea truncatula has been an electrophoretic separation of mucus (Wilson, 1968b). Fortunately neither the determination of total protein nor the electrophoretic work presented any problems with the small amounts of haemolymph available.

For the determination of total protein, the very sensitive colorimetric method of Lowry, ~~et al~~ Rosenbergh, Farr, and Randall, (1951) was used. From other

work (e.g. Roach, 1963; Trams et al, 1965) the level of total protein in haemolymph was expected to be between 10 and 20 mg/ml, with the calculation in Part III 11) suggesting a level of 15.86 mg/ml. A protein stock solution was made up by diluting 5 ml fresh Wellcome chemical control serum to 500 ml with distilled-deionised water. This stock solution contained 700 ppm protein, and was stored at $4^{o}C$ for a maximum of 2 days. A mixed protein solution of this type was thought to provide more suitable standards than, say, a pure solution of albumin.

The colorimetric procedure was first tried, setting up duplicate tubes containing from 0.05 to 0.30 ml protein stock solution and adding 0.95 to 0.70 ml distilled-deionised water to give a sample volume of 1 ml in each tube. Duplicate blanks were set up containing 1 ml distilled-deionised water. The tubes used were wide diameter pyrex boiling tubes. To each tube was added 5 ml of the 'reagent mixture'. This was prepared fresh by mixing 1 ml of 1% aqueous $CuSO_4 \cdot 5H_1O$ (Analar) with 1 ml aqueous sodium tartrate and diluting to 100 ml final volume with an aqueous solution containing 20 gm Na_2CO_3 (Analar) and 4 g NaOH (Pronalys pellets) per litre. The tubes were left to stand for 10 minutes. Folin-Ciocalteau reagent (B.D.H.), stored at $4^{o}C$

was freshly diluted 1:3 with distilled-deionised water, and 0.5 ml diluted reagent was added to each tube in turn followed by 2 minutes vigorous mixing on the Whirlimixer (Fisons). Thus the final volume was 6.5 ml. The tubes were left to stand for 30 minutes and then the colours were read at 670 mμ on a Gallenkamp Mk 3 bench colorimeter using the heat filter ON22 and the Ilford colour filter 608. Table 12.a.) (Appendix III) gives the readings. The actual weights of protein used were 35, 70, 105, 140 and 210 μg.

An identical procedure was used for haemolymph samples, which were all collected using carbon dioxide during anaesthetisation. The first set of samples used comprised one 10 μl sample and 2 5 μl samples, all three being taken from larger pooled, centrifuged volumes. This was to show whether or not a 5 μl would give adequate colour development. The second set of samples were all of 5 μl volume, and duplicate 70 and 140 μg standards were run concurrently with both sets. Colour development and reading was as described above, and Table 12.b.) (Appendix III) gives the readings.

For the electrophoresis, a Gelman tank and Shandon power supply were used to obtain separations on cellulose acetate strips

in pH 8.6 barbitone (veronal) buffer. The strips were soaked in buffer for at least 10 minutes before use, and were on occasions left for up to 7 days. The Gelman tank was filled with 500 ml fresh buffer and the volumes in the anodic and cathodic chambers equalised by tilting the tank. The strips were removed from the soaking buffer solution and superficially dried on filter paper. Spade-ended forceps were used to handle the strips. Freshly-collected centrifuged haemolymph - collected using carbon dioxide during anaesthetisation - was taken as required from a 30 µl pooled sample. Both strip and spot applications were tried, the former using a Shandon bar applicator filled from a 5 µl micro-pipette, and the latter using a 1 or 2 µl pipette directly. Haemolymph samples were applied quickly, without letting the strips dry out. The strips were then quickly positioned in the tank and held in place by magnetic holders ('Magnagrips'). The tank was then closed and electrophoresis commenced. The time/voltage combinations used were: 30 minutes at 300 volts; 60 minutes at 255 volts; and 90 minutes at 210 volts. After switching off, the strips were carefully removed and treated with a location reagent. This was made up by dissolving 50 g hydrated Zinc sulphate (Analar), 0.1 g bromophenol blue (B.D.H.) and 50 ml

glacial acetic acid (Analytical grade), in 1 litre distilled-deionised water. The strips were submerged in the location reagent for 10 to 30 minutes at room temperature and then rinsed with 2% acetic acid to remove excess stain. Final colour development was by rinsing the strips in tap water, stopping the procedure by transfer to distilled water. It was found to be very easy to over-develop the colours and to 'blue' the whole strip. Strips were preserved for photography (see Plate 9) by rinsing well in distilled water and storing in a humidity chamber. Human plasma, donated by the author, was run concurrently with the haemolymph samples to provide a known standard.

Results

For the colorimetric procedure, good linearity was proved (Table 12.a.) (Appendix III)) up to around 150 µg protein although the readings for the 35 µg standards appear a little high. From the first set of determinations the level of protein in haemolymph was calculated to be 13.95 mg/ml, and from the second set 13.38 mg/ml. With such few repeats the level of total protein in haemolymph is best defined as being between 13 and 14 mg/ml. This is tolerably close to the value predicted from nitrogen determinations.

Plate 8. shows the results of a typical electrophoretic separation, but in general the strips did not preserve well for photography. The black spots and dotted lines represent origin points. Results were generally poor but did show that there were 2 main protein fractions in haemolymph, with trace of a minor component sometimes showing between them. The haemolymph proteins showed mobility comparable with the α-albumin and γ-globulin fractions of human plasma. The distances moved from anode to cathode from the origin points were: Haemolymph fraction 1, 40 mm; Haemolymph fraction 2, 34 mm: human albumin, 44 mm; human γ-globulin, 34 mm; human β-globulin, 32 mm; human α_2-globulin, 28 mm; human α_1-globulin, 21 mm, for a typical run at 255 volts for 60 minutes. The various time/voltage combinations used did little to improve separation. A more refined technique would probably have revealed sub-fractions of the 2 main haemolymph proteins.

The results of total protein determinations and the electrophoretic separation are compared with data for other species in the discussion and summary at the end of Part III.

14) The determination of lipids in haemolymph

Methods

There is at present virtually no information available on the lipid compounds found in pulmonate molluscs. Fox (1966) reviews work on fat-soluble pigments, including carotenoids, but none of the analytical work cited elsewhere in Part III includes any information on lipid fractions. Wilson (1968b), however, gives useful information on the lipids found in mucus from Lymnaea truncatula; identifying and determining many types of lipids, including β-carotene, presumably from the algal diet. Similar methods to those used by Wilson (1968b) were used here, but a detailed analysis of lipids was not attempted. With such small amounts of material available it was felt that the difficulty in even identifying specific lipids was immense and that a complete analysis of, say, phospholipids, would be impossible. Also it was realised that even if a complete analysis were possible it would be extremely difficult to add the required amounts of each lipid to an artificial haemolymph, as many of the molecules involved are insoluble unless bound to protein.

A brief analysis using thin layer chromatography was first attempted and the techniques used are fully described by Stahl

(1965). Ethanol/ether extracts of 10 and 20 µl pooled haemolymph samples were used, with standard solutions of tristearin, oleic acid, cholesterol, and lecithin, all dissolved in A.R. chloroform. The Ethanol/ether extraction solvent was 3:1 by volume in composition, and was heated to 60°C. Silica-gel G plates were used with hexane: ether: acetic acid (90:10;1) solvent, and spots were located with iodine vapour.

A quantitative determination was made of the cholesterol + cholesterol esters in haemolymph, as this was the only lipid fraction specifically identified (see Plate 9). The colorimetric method used depends on the Liebermann-Burchard reaction and is widely used in clinical work (e.g. Watson, 1960; Lund, Gill, and Andreen, ~~et al~~, 1961). The reagents used were:

1) A solution of acetic acid and acetic anhydride - 6.4M and 7.0M respectively, made up with A.R. reagents.
2) Concentrated sulphuric acid (Analar).
3) Glacial acetic acid (A.R.).
4) A stock solution of cholesterol (B.D.H.) containing 4 mg/ml in glacial acetic acid.

5 pooled 20 µl haemolymph samples, collected using carbon dioxide during anaesthetisation, were first extracted for cholesterol esters in glass-stoppered tubes. The solvents used were 3:1 ethanol: ether for 1 hour, and pure ether for a further 2 hours, and the tubes were held at 60°C in a shaking water bath during extraction. After each hour the samples were centrifuged and supernatants collected in 5 micro-kjeldahl flasks. The pooled extracts were evaporated to dryness over the 60°C water bath using the filter pump to provide reduced pressure. The first set of haemolymph extracts were ruined by using a rubber bung attachment on the filter pump tube. Although previously washed repeatedly in ether and heptane, the bung caused contamination of extracts during drying as it was extracted itself by ether condensing on its lower surface and running back into the flask. On several occasions small rubber fragments dropped off to contaminate samples very heavily. Thus glass or silicone bung attachments were used instead.

Dry extracts were taken up in 20 µl glacial acetic acid and then 1 ml of the acetic acid/acetic anhydride solution added, directly into the micro-kjeldahl flasks. Triplicate 20 µl blanks of glacial acetic acid alone, and triplicate 20 µl cholesterol

standards, containing 10, 20 and 40 µg cholesterol, were treated similarly. The latter were made up fresh by dilution of the stock solution with glacial acetic acid. The flasks were then capped and left to stand for 15 minutes. 0.2 ml concentrated sulphuric acid was then added to each flask in turn, mixing by cautious tapping. 15 minutes were allowed for colour development, before reading undiluted at 575 mµ on the Hilger-Watts Uvispek spectrophotometer in 0.2 ml capacity micro-cells. The instrument settings were: sensitivity, 8; input sensitivity, x 5; slit width, 0.05 mm; and the readings are given in Table 13 (Appendix III).

Results

, Plate 10 shows a typical chromatogram for 10 µl and 20µl haemolymph samples. Phospholipids, cholesterol, free fatty acids, and a triglyceride fraction are all shown. The huge spots nearest the solvent front are possibly cholesterol esters, but could also contain the β-carotene fraction which is probably eventually deposited in the body wall and mucus (Wilson, 1968b). Cholesterol itself was seen as a discreet spot in all chromatograms.

From the dats in Table 13 (Appendix III) the haemolymph

extracts contained 15.35, 16.75, 17.44, 20.58 and 24.07 µg cholesterol + esters. The mean of these results is 18.84 µg (± 3.13 standard deviation), and since the extracts were from 20 µl samples, the level of cholesterol + cholesterol esters in haemolymph was found to be 942 ± 157 ppm. This is a very high result as Wilson (1968b) found only 2.0 to 2.5 µg cholesterol per mg of mucus. In clinical determinations, the usual level of cholesterol in serum is 1200 to 2300 ppm., and levels above 2500 ppm are regarded as pathological. The above analysis does at least show that haemolymph contains many different type of lipid which may or may not eventually prove essential constituents of the environment of larval trematodes.

15) Analytical methods which were found to be unsuitable for use with haemolymph.

1) The methods of Lowry and Hastings (1942) and Smith et al (1954) for the colorimetric determination of potassium were tried without success. Standard K^+ solutions and haemolymph samples both failed to give precipitates with chloroplatinic acid.

2) The method of Williams and Wilson (1961) for the colorimetric determination of calcium was tried using the author's own preparation of glyoxal-bis-2-hydroxyanil. Colour development was good, but the colours were extremely unstable.

PART III Discussion and Summary

The main constituents of the haemolymph of Lymnaea truncatula have been determined with tolerable accuracy. The results of the analysis are summarised below, and compared with data for other species. Other analytical work on haemolymph is reviewed by Florkin (1954), Robertson (1964), Goddard and Martin (1966), and Florkin (1966).

Table 7. Mean values for inorganic ions, pH and measurements (concentrations in ppm).

Measured	1) Lymnaea truncatula	2) Helix pomatia	3) Arion ater	4) Cepaea nemoralis	5) Lymnaea stagnalis	6) Lymnaea stagnalis	7) Planorbis corneus
Na^+	1126	1449	1426	1978	1090	718	1976
K^+	94.8	164	105.3	170	109.2	46.8	89.7
Ca^{++}	165	412	112	47	120	284	240
Mg^{++}	50	321	138	16.4	117	51	26.7
Cl^-	1141	2556	1862	2276	1512	966	-
HCO_3^-	1120	1280to1650 *	1513	1403	-	-	-
SO_4^{--}	-	-	212	50	-	-	30
PO_4^{---}	7.6	-	14.9	50	-	-	-
pH	8.02	7.6to8.8*	8.83	7.92to8.32	-	-	-
$\Delta\,^{\circ}C$	0.280	0.3to0.4*	0.371	-	-	-	-

References: 1) The present study 2) Duchâteau and Florkin (1954) 3) Roach (1963) 4) Trams et al (1965) 5) Huf (1934) 6) Duchâteau and Florkin (1954) 7) Florkin (1943).

* Cites Duval (1930) for HCO_3^- and $\Delta^{o}C$, and Roach (1963) for pH values from various authors.

Other useful information for comparison is given by Burton (1965) who compares the levels of Na^+, K^+ and Mg^{++} in mucus and haemolymph of Helix aspersa. Picken (1937) gives the only Δ measurement for a Lymnaea sp. i.e. $0.25^{o}C$ for Lymnaea peregra. Table 7. and the other literature cited show the wide differences in haemolymph composition between species, and confirms the original need for an analysis of Lymnaea truncatula haemolymph. Also apparent is the variability of haemolymph within a species, according to the individual snails environment, nutrition, and activity. This is particularly seen in pH and Δ measurements, (e.g. Duval, 1930; Kamada, 1933; Arvanitaki and Cardot, 1932; Roach 1963). The figures given for Lymnaea stagnalis in Table 7. are an illustration of the discrepancies between the results of different authors. Generally speaking, the present work on Lymnaea truncatula has produced no surprises, and the levels of ions determined fall roughly in between

those known for fully aquatic and fully terrestrial snails. This is very encouraging and it should be noted that in no case has a ridiculous figure been obtained for any component to suggest any severe effects from anaesthetisation. One rather disturbing factor is, however, the imbalance between anions and cations. These total approximately 50 and 64 mEq/litre respectively, and the fact that sulphate was not determined could only account for a small portion of this discrepancy. In most other fairly complete analyses (e.g. Trams et al, 1965) there is a similar imbalance of cations over anions and this is said to be accounted for by the anionic effect of proteins and organic acids. The present imbalance in Lymnaea truncatula is rather too large to account for by this reasoning, and is impossible to explain fully. Indeed Roach (1963) has produced an almost perfectly balanced analysis for Arion ater.

Table 8. Mean values for organic components, excluding amino acids (concentrations in ppm except for 2.): + denotes present

Compound	1) Lymnaea truncatula	2) Lymnaea truncatula	3) Cepaea	4) Arion ater nemoralis
Glucose	814	0.25 gm/mgm	240	-
Total protein	13500	-	22200	10550
No. of protein fractions	2 or more	5 or more, but only 2 soluble*	-	-
Organic phosphorus	2.55	-		

(*see foot of Page 142)

	1)	2)		3)	4)
Compound	Lymnaea truncatula	Lymnaea truncatula		Cepaea nemoralis	Arion ater
Cholesterol + esters	942	1.95to2.46	gm/mgm	27.9	-
Phospholipids	+	+		-	-
Triglycerides	+	+		-	-
Free fatty acids	+	0.003to0.007	gm/mgm	-	-
Cholesterol esters	+	+		-	-
Esterified fatty acids	?	0.004to0.006	gm/mgm	-	-
-Carotene	?	+		-	-
Total nitrogen	2692	-		-	-
Non-protein nitrogen	145.5	-		-	-

References: 1) The present study 2) Mucus analysis (Wilson 1968b) 3) Trams et al (1965) 4) Roach (1963).

Table 8. shows the general lack of knowledge of the organic constituents of haemolymph, and also reveals the gaps in the present work. Carbohydrate metabolism is reviewed by Goddard and Martin (1966) and nitrogen metabolism by Florkin (1966). The electrophoretic work on proteins gave little useful information, but it is interesting that Wilson (1968b) found only 2 protein fractions in aqueous extracts* of

mucus, which with a similar buffer system behaved very like the 2 main fractions found in haemolymph. This tends to support Burton's (1965) concept of haemolymph playing a large part in mucus production. It is possible that the above organic analysis is inadequate for developing a culture medium for larval Fasciola hepatica.

Table 9. Mean values for amino acids (concentrations in ppm, except for 2) which are in µgm/gm; + denotes 'present')

Amino acid	1) Lymnaea truncatula	2) Lymnaea truncatula	3) Lymnaea stagnalis	4) Helix aspersa	5) Biomphalaria glabratus a	6) Planorbis corneus
Cysteic acid	1.93	-	-	+	-	-
Hydroxy-l-proline	+	-	-	-	-	-
Aspartic acid	6.94	36.3	-	5.32	2.80	+
Threonine	18.45	25.0	7.2	+	3.10	+
Serine	24.22	42.7	3.7	15.77	5.99	+
Glutomic acid	23.87	179.0	0.4	8.83	4.71	+
Proline	(65.11)	45.9	1.6	+	2.88	+
Glycine	9.58	60.4	2.7	12.01	2.40	+
Alanine	40.80	95.4	2.1	26.27	2.41	+
∝-amino-n-butyric acid	2.82	-	0.9	+	-	-
Valine	26.14	35.3	+	12.89	1.64	+
Cystine	5.65	11.8	+	-	-	+
Methionine	6.33	3.1	-	-	0.15	+
Isoleucine	8.15	21.1	1.1	-	1.05	+

Amino acid	1) Lymnaea truncatula	2) Lymnaea truncatula	3) Lymnaea stagnalis	4) Helix aspersa	5) ~~Australorbis~~ Biomphalaria glabrata	6) Planorbis corneus
Leucine	11.03	27.5	1.4	7.87	1.44	+
Tyrosine	+	+	0.6	+	+	+
Phenylalanine	+	-	1.8	-	0.92	+
Ornithine	+	27.7	0.5	-	-	-
Lysine	+	6.2	2.1	-	2.96	+
Histidine	+	6.5	1.9	+	2.02	+
Arginine	-	304.5	-	+	1.22	+
Trytophan	-	-	4.1	-	-	+
Asparagine	-	+) 5.5		-	-	-
Glutamine	-	-) total		44.20	-	-
Urea	-	-	-	-	-	-
Ammonia	5.29	+	-	+	-	-

References: 1) The present study 2) Mucus analysis (Wilson, 1968b) 3) Friedl (1961c) 4) Kerkut and Cottrell (1962) 5) Gilbertson et al. (1967) 6) Targett (1962b)

The above literature and information given by Dusanic and Lewert (1963), Targett (1962a), and Florkin (1966) shows the wide differences in amino acids levels between species,. The present analysis appears fairly complete, but the fact that arginine, glutamine, trytophan, and asparagine were not found should not be taken as conclusive proof of their absence, as the separation system used was not a well-tried technique. Most of the determined levels appear acceptable - with the possible exception of

proline - and in general levels are near to those found by Kerkut and Cottrell (1962) for Helix aspersa. Cheng (1963) discusses the biochemical requirements of larval trematodes and it appears that the array of amino acids determined here should be adequate for larval Fasciola hepatica.

It is interesting at this point to sum the dry weights of the determined components of haemolymph, and to compare the result with the determined dry weights. The mean determined dry weight of haemolymph was 18.80 mg/ml, but this takes no account of bicarbonate which was destroyed by heating. The summed value for all compounds is 19.23 mg/ml, which reduces to 18.67 mg/ml when bicarbonate is deducted. Thus the figures show very satisfactory agreement.

As is repeatedly mentioned in the text, haemolymph samples were both centrifuged and pooled for most determinations: the former to remove cells and the latter to provide adequate sample volume. In general the accuracy of determinations was good, but it is unfortunate that pooling was always necessary and that no work to show variation between individual snails was possible. The analysis has obvious gaps and discrepancies (e.g. the anion-cation imbalance). However, the checks on determinations e.g. nitrogen values/total protein and dry weight/total solids, are

satisfactory and the determined value for Δ appears realistic. The gravest omissions from the analysis are 1.) sulphate determinations. 2) Information on trace cations e.g. Fe, Cu, Mn. 3) Detailed information on proteins and lipids. 4) Information on growth factors such as vitamins.

With the amounts of sample material available, 2) and 4) are virtually impossible. The information obtained is certainly sufficient to develop a very useful physiological saline either for handling larval Fasciola hepatica or for tissue explants from Lymnaea truncatula. The shortcomings of the analysis as a basis for a culture medium are discussed in Part IV.

Plate 5. Apparatus for melting-point determinations.

a) A view of the melting-point apparatus with the battery-driven stirer, Beckmann thermometer and viewing microscope all in position. The 'thermos' flask holds liquid nitrogen.

b) A view of the interior of the apparatus, showing the glass alcohol bath, sample carrier, and cotton wool insulation.

a)

b)

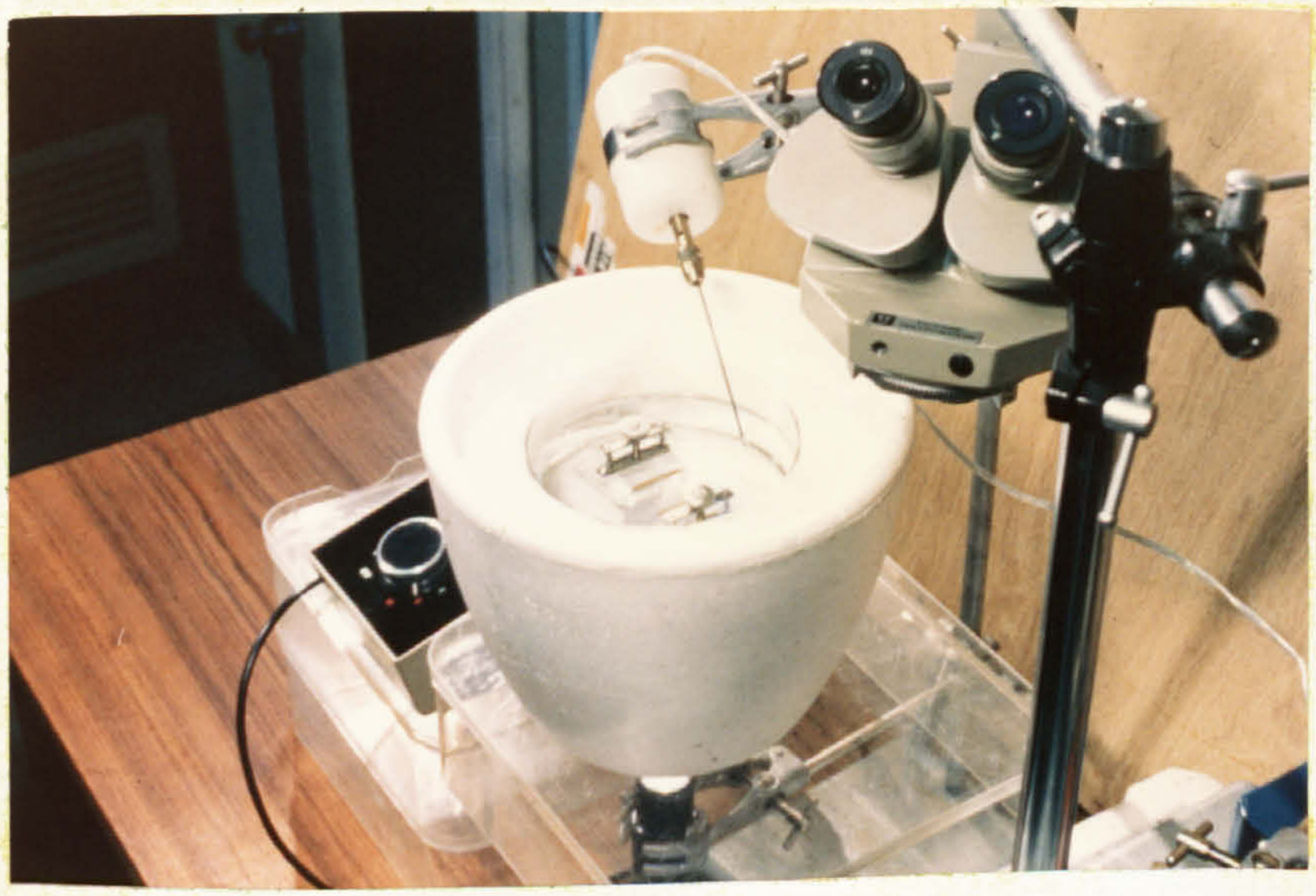


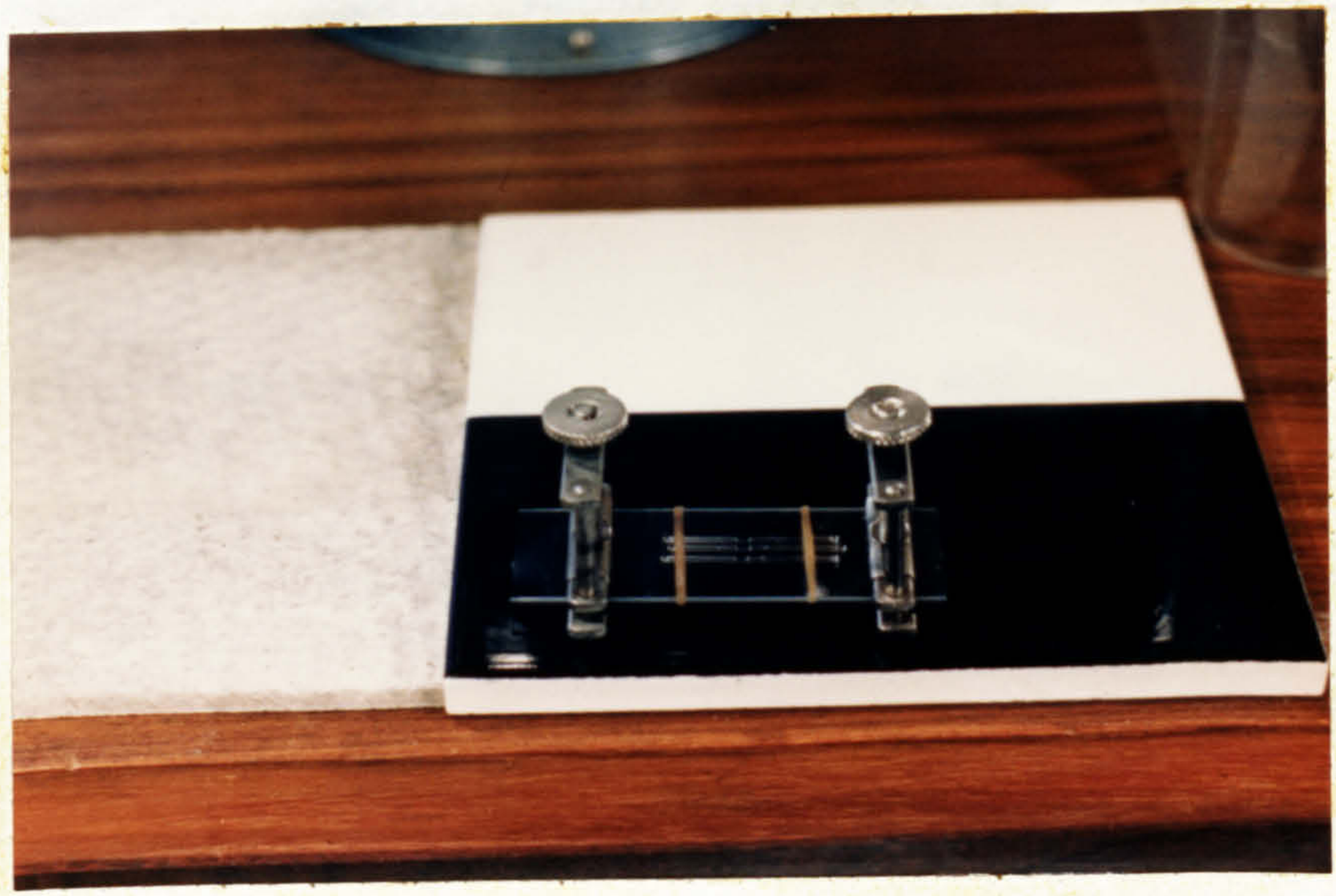
PLATE 5

Plate 6. **Apparatus for melting point determinations**

a) A view of the sample carrier showing capillary tubes held in place with rubber bands, and the screw-clamps which provide weight and stability.

b) A view of a frozen sample in polarised light.

a)



b)

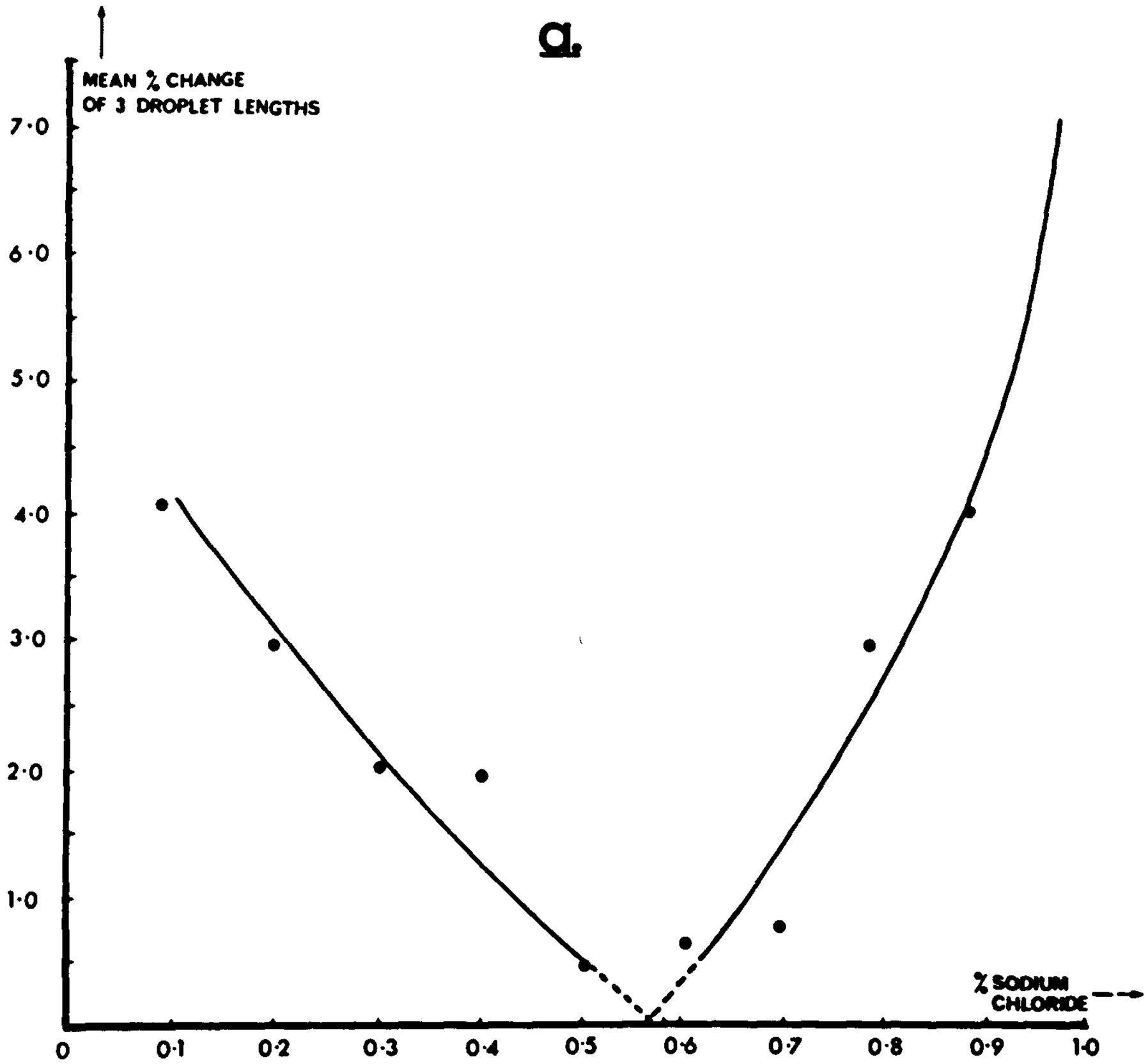
PLATE 6

Figure 2. Osmotic pressure determinations: capillary method.

a) Graph relating change of droplet length to concentration of bordering drops of NaCl solution, for the vapour pressure method of determining osmotic pressure.

b) The arrangement of drops in the capillary tubes used for the vapour pressure method of determining osmotic pressure.

a.


MEAN % CHANGE
OF 3 DROPLET LENGTHS
7·0
6·0
5·0
4·0
3·0
2·0
1·0
% SODIUM
CHLORIDE
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0

b.


STANDARD
SAMPLE
STANDARD

FIGURE 2

Figure 3. The melting-point apparatus.

1. Beckmann thermometer.
2. Polystyrene container and lid.
3. Cotton wool insulation.
4. Polystyrene light tunnel.
5. Polaroid 1. and heat filtering glass.
6. Microscope light.
7. Solid watch glass.
8. Sample carrier
9. Alcohol bath.
10. Stirrer.
11. Polaroid 2.
12. Viewing microscope.

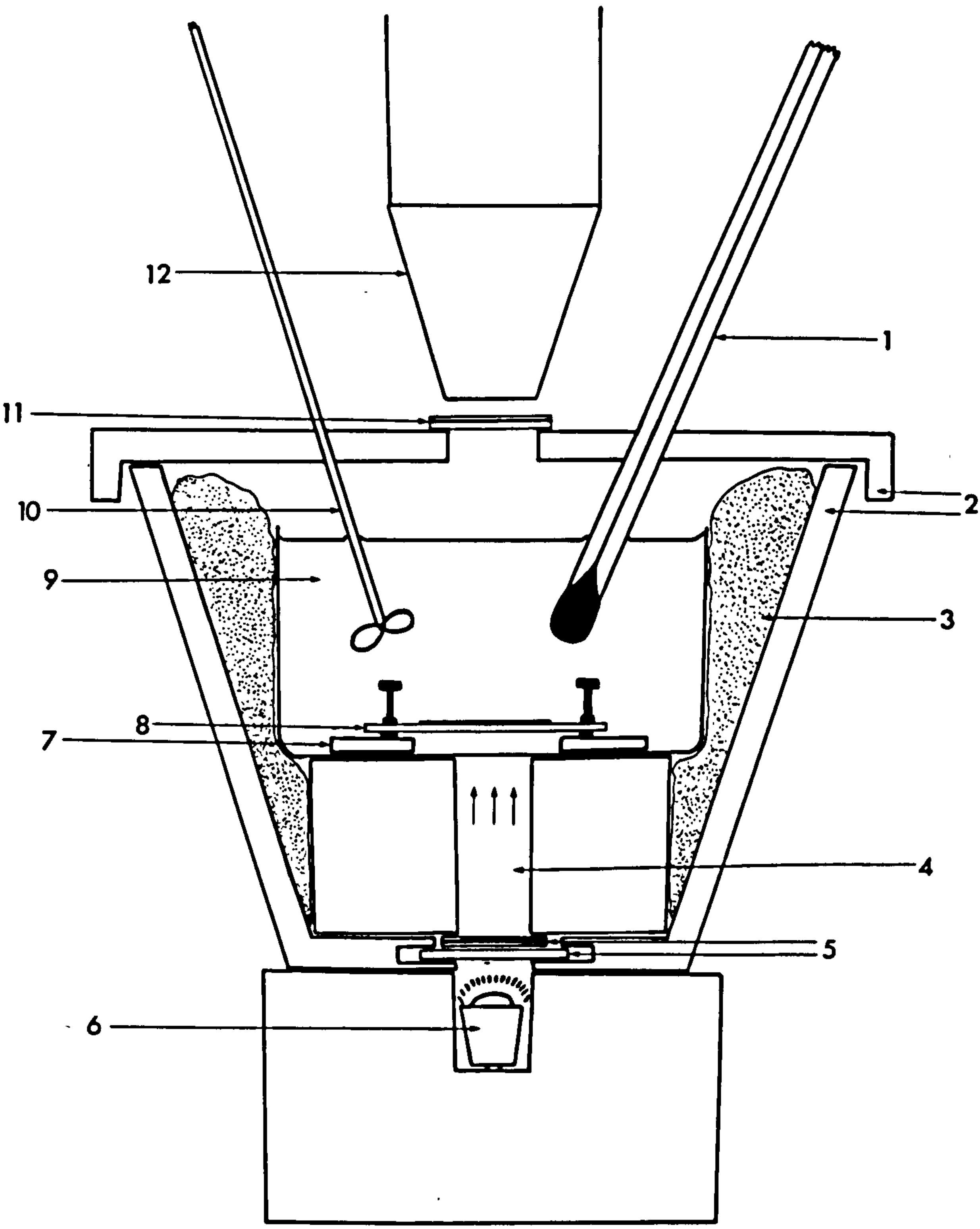


FIGURE 3

Figure 4. Calibration curve for the flame photometric determination of Na^{+}, K^{+}, Ca^{++} and Mg^{++}

a) Sodium.

b) Potassium.

c) Calcium.

d) Magnesium.

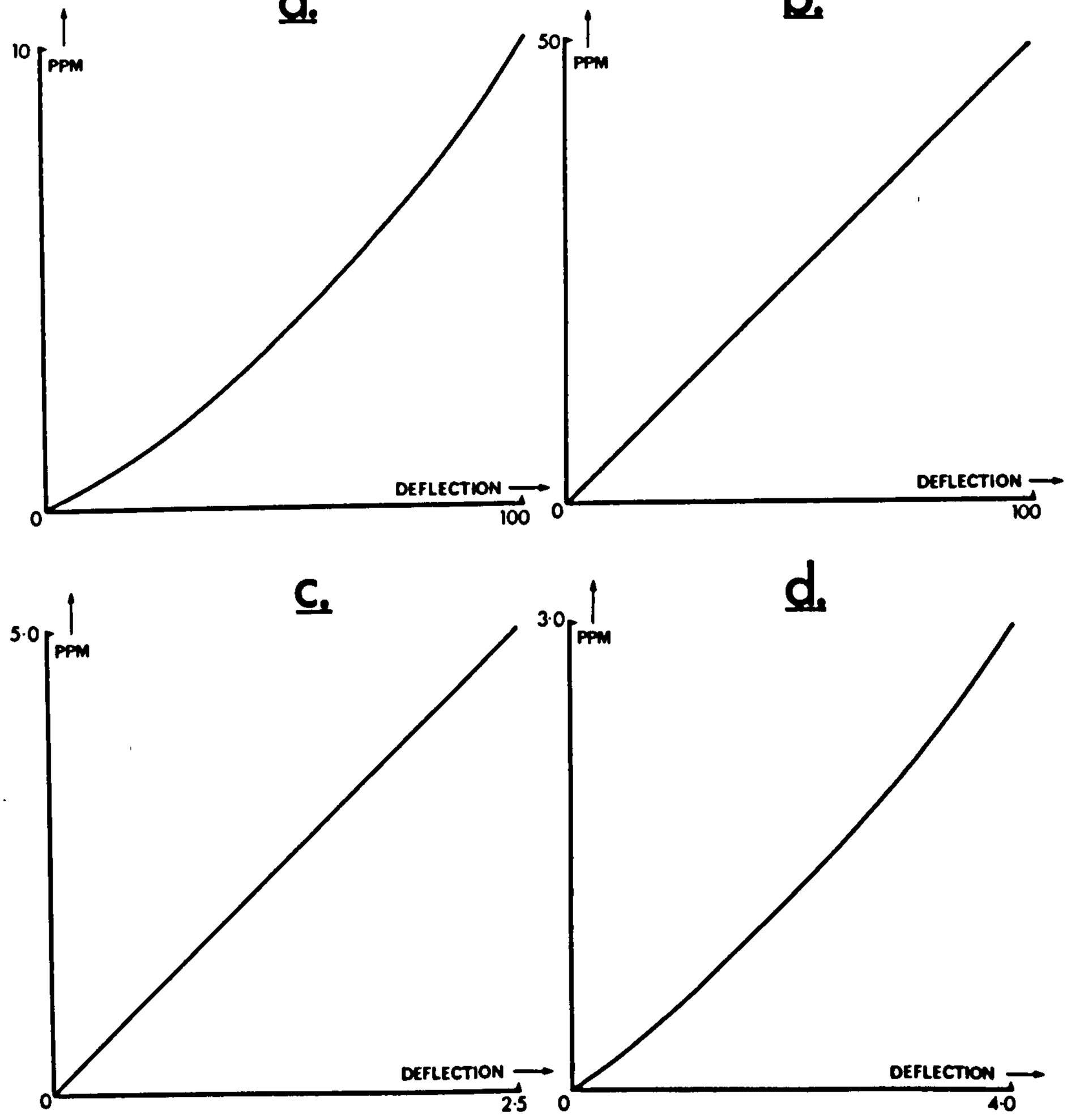

a.
10
PPM
DEFLECTION
0
100
b.
50
PPM
DEFLECTION
0
100
c.
5·0
PPM
DEFLECTION
0
2·5
d.
3·0
PPM
DEFLECTION
0
4·0

FIGURE 4

Figure 5. A conductivity cell.

1. 1 μl micropipette introducing HCl.
2. Sample droplet.
3. $Ba(OH)_2$ solution.
4. Electrodes.
5. Araldite plug to prevent fracture of electrodes.
6. Leads to conductance bridge.
7. Siliconised side walls.
8. Coverslip lid.

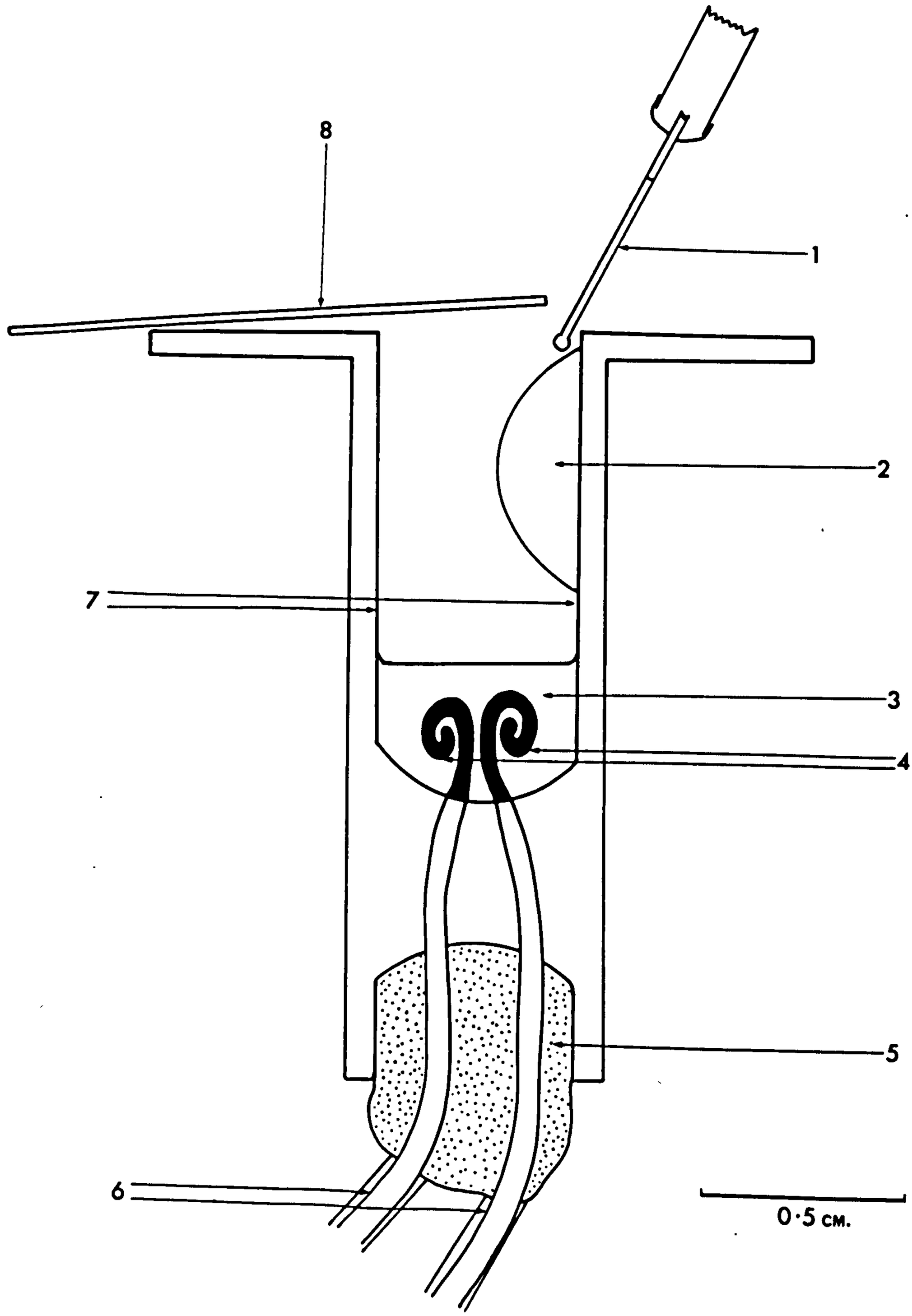


FIGURE 5

Figure 6. **Conductivity/time graphs.**

Three conductivity/time graphs for a 1500 ppm HCO_3^- standard solution.

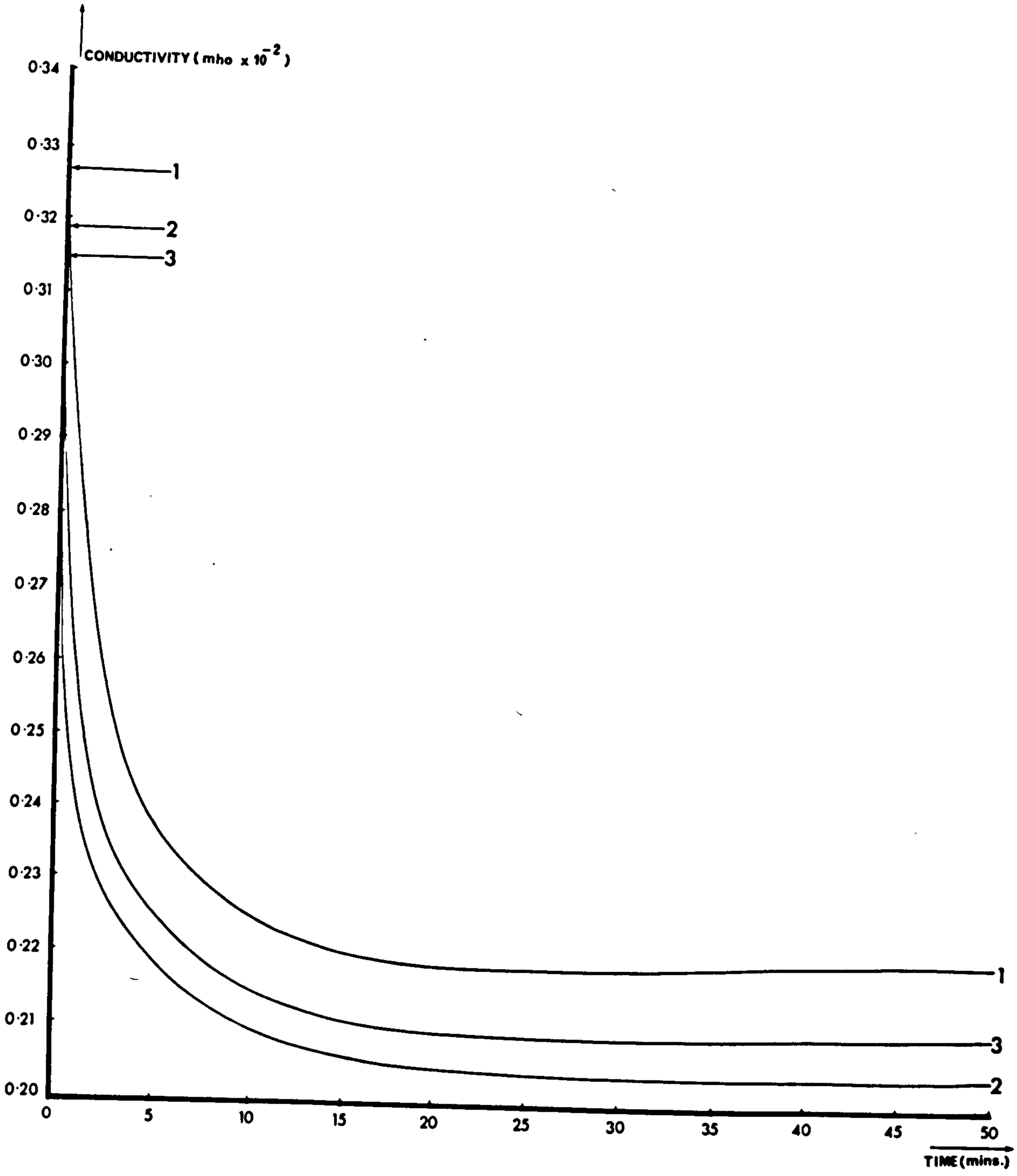

CONDUCTIVITY (mho x 10^{-2})
0·34
0·33
0·32
0·31
0·30
0·29
0·28
0·27
0·26
0·25
0·24
0·23
0·22
0·21
0·20
1
2
3
0
5
10
15
20
25
30
35
40
45
50
TIME (mins.)

FIGURE 6

Plate 7. **A Conductivity Cell.**

PLATE 7

Plate 8 Carbohydrate chromatography

Plate 8 Chromatogram

A typical chromatogram showing the glucose content of haemolymph. (called serum in this case).

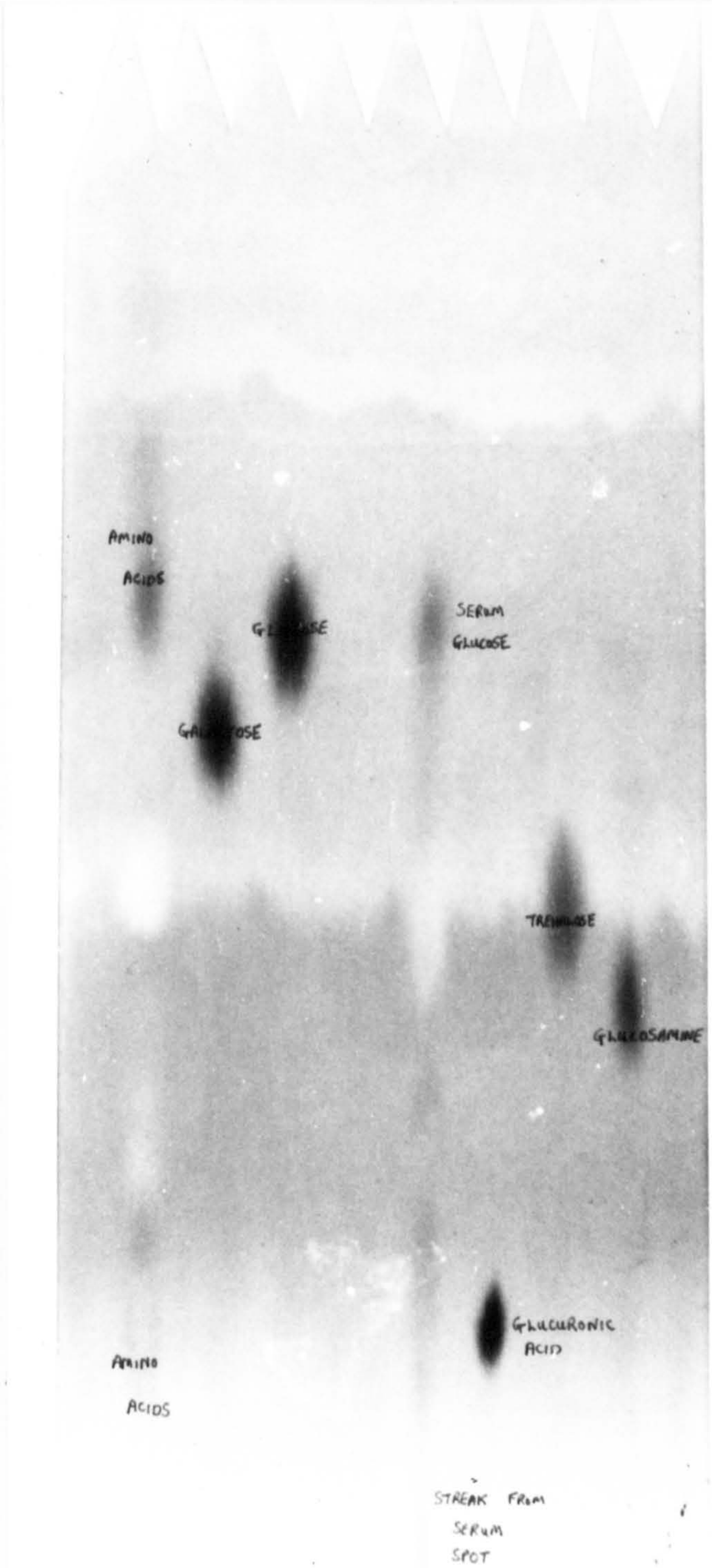

AMINO
ACIDS
SERUM
GLUCOSE
TREHALOSE
GLUCOSAMINE
GLUCURONIC
ACID
AMINO
ACIDS
STREAK FROM
SERUM
SPOT

PLATE 8

Plate 9. **Electrophoresis of haemolymph proteins.**

Electrophoresis strips: the upper three strips show separations of haemolymph with 2 strip applications and one spot application and the dotted lines and large black spot represent origin points. The bottom strip shows a spot application of human plasma.

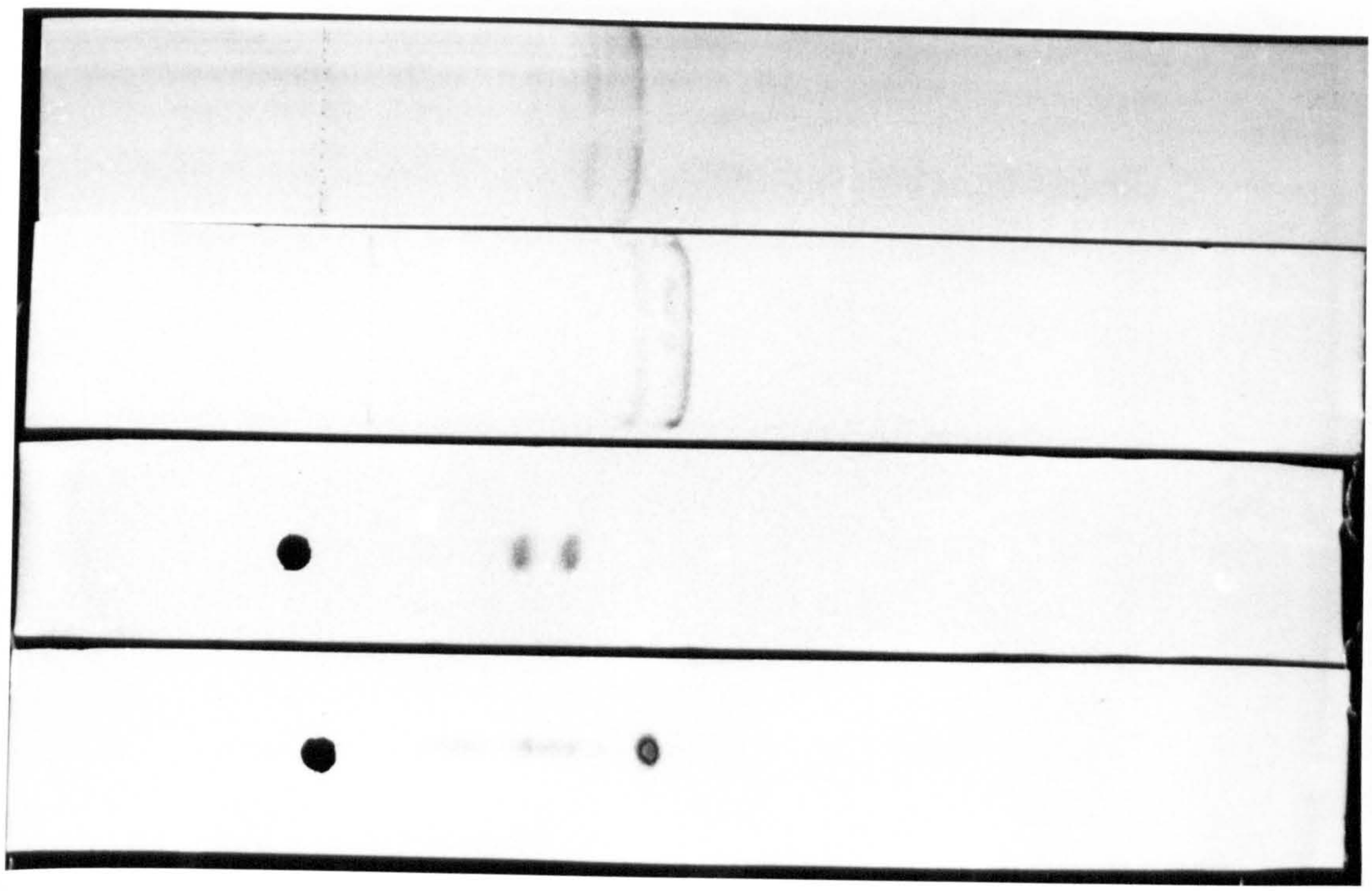


PLATE 9

Plate 10. A typical thin layer chromatogram of haemolymph lipids.

The spots applied were:

1. Cholesterol.
2. Tristearin (faint).
3. Lecithin.
4. Oleic acid.
5. 10 μl. extracted haemolymph.
6. 20 μl. extracted haemolymph.

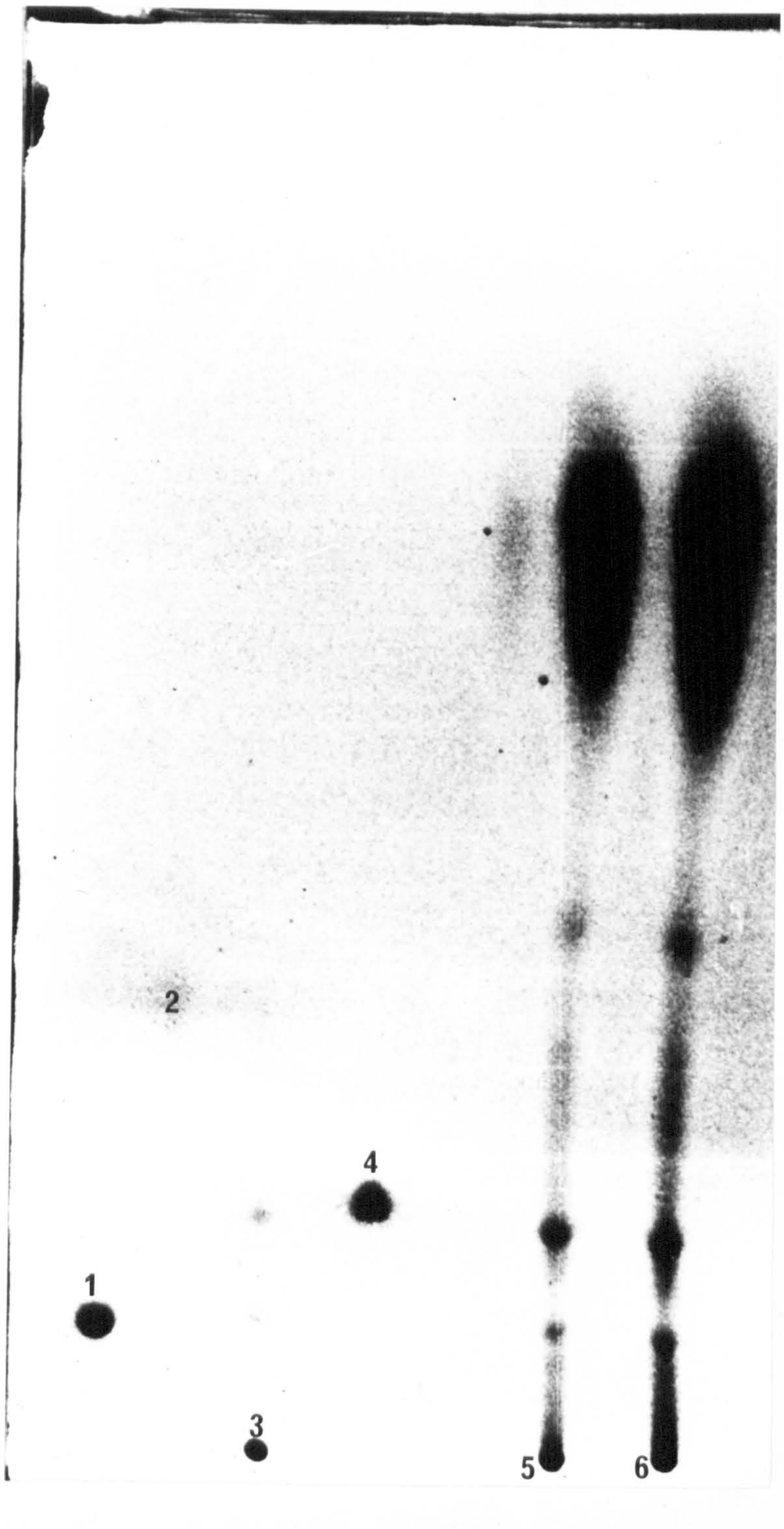


PLATE 10

PART IV The development of a basic culture medium.

Introduction

The analysis described in Part III provides sufficient information to devise a medium similar to the haemolymph of Lymnaea truncatula in ionic composition, pH, and tonicity. The development of a culture medium for parasites is a difficult task (Taylor and Baker, 1968), and it was felt here that the basic necessity was to keep the medium fully defined and as simple as possible (see Introduction). Paul (1965) discusses defined media used in cell and tissue culture, and the medium described here is constructed along similar lines. A balanced saline for Lymnaea truncatula is first described and then addition of amino acids and vitamins to produce a more complete medium is discussed. Stock solutions are described which allow rapid preparation of large volumes of medium. The validity of a protein-free and lipid-free medium as an environment for larval Fasciola hepatica is discussed.

Methods

1) Devising a balanced saline.

The imbalance between determined anions and cations in haemolymph presented some difficulty in devising a balanced saline. This, and the omission of protein from the basic culture medium meant that

the levels of Cl^- and HCO_3^- had to be higher than those determined for haemolymph (see Part III discussion). Solubility considerations dictated the compounds chosen and a basic scheme was devised to supply Na^+ and K^+ as bicarbonate salts, and Ca^{++} and Mg^{++} as chlorides. This (see results below) allowed the levels of Cl^- and HCO_3^- to be raised in proportion above those determined for haemolymph, to balance the cations. Phosphate was incorporated as $Na_2HPO_4 \cdot 12H_2O$. In retrospect this was probably a mistake as the use of Na PO instead would have given complete dissociation of the salt and a more reliable level of PO_4^{---}. The amount of $Na_2HPO_4\ 12H_2O$ used was calculated to contain PO_4^{---} equivalent to the <u>total</u> PO_4^{---} determined for haemolymph. This was because it was felt that both free and bound phosphate is probably available to larval <u>Fasciola hepatica</u> bathed in haemolymph, and with such low phosphate levels anyway, Overestimation of the amount needed was better than underestimation. The phosphate level calculated was far too low to cause any precipitation problems (Paul, 1965). An arbitrary amount of sulphate was added as $(NH_4)_2\ SO_4$, calculated from the level determined by Florkin (1943) for <u>Planorbis corneus</u>. Glucose was included to complete the balanced saline. The results 1.) below give the amounts of compounds calculated to make 1 litre of balanced saline and the amounts of individual ions provided. A procedure is described which prevented precipitation when making up the balanced saline. The pH of the balanced saline was determined.

2) A basic culture medium, based on the balanced saline.

No attempts were made to introduce proteins or substitute macromolecules. Attempts were made to introduce cholesterol both by direct dissolution and by the addition of small volumes of concentrated solutions in various organic solvents. These attempts proved fruitless and it was decided that the basic medium should be kept protein-free and lipid-free. Lipid molecules are in any case probably best introduced into aqueous media bound to proteins.

The incomplete nature of the analysis of haemolymph amino acids is discussed in Part III. The amino acid levels that were successfully determined appear low compared with those of invertebrate cell and tissue culture media (Paul, 1965). These media were, however, developed for insect tissues and are of no use in suggesting levels for those amino acids not determined in the haemolymph analysis. After comparing the levels determined for haemolymph amino acids with those used in many defined media (Paul, 1965), it was decided that medium NCTC 109 (Evans et al, 1956 - see Paul, 1965) contained amino acids in very similar concentrations. This medium is a very complex defined medium containing a full complement of amino acids, vitamins and other organic molecules. Thus it was decided that, with a few exceptions, amino acids not quantitatively determined should be added empirically

to the basic culture medium at the levels used in NCTC 109. Vitamins were also added on the same basis. Exceptions to this rule were phenylalanine, lysine, ornithine, and histidine which were added empirically to give a final concentration of 10 mg/litre, and nicotinic acid and antibiotics for which levels were taken from Eagle's Medium, 1959, as given by Paul (1965). A more realistic level for Proline (see PartIII) was calculated by comparing the original chromatograms with some new runs on standard amino acid mixtures, refering the areas of the proline peaks to those of glycine standards.

Other trace components of NCTC 109, such as coenzymes, were not included, but glutamine, a major component of many media (Paul, 1965) was included. Cysteine hydrochloride is also present as a major component of NCTC 109 and was included in the medium developed here as a safeguard against possible underestimation of cystine, and to offset the general lack of sulphur-containing molecules.

Thus a basic culture medium was devised by adding to the balanced saline the determined levels of amino acids in haemolymph and empirical amounts of non-determined compounds. A system of stock solutions was worked out to enable batches of medium to be made up very rapidly. The scheme followed that for Eagle's medium, 1959. The preparation and storage of stock solutions is discussed by Paul (1965). The

results 2) below give the full composition of the basic medium (designated BCM) and give details of the preparation, sterilisation and storage of stock solutions.

Results

1) Devising a balanced saline

Table 10a Weights of compounds calculated to make 1 litre of balanced saline

	Compound	Weight (g)	Weights of each ionic component supplied (g).								
			Na^+	K^+	Ca^{++}	Mg^{++}	NH_4^+	Cl^-	HCO_3^-	PO_4^{---}	SO_4^{--}
1	NaCl	1.812	0.712					1.100			
2	$NaHCO_3$	1.500	0.411						1.089		
3	$KHCO_3$	0.243		0.095					0.148		
4	$CaCl_2 \cdot 2H_2O$	0.605			0.165			0.292			
5	$MgCl_2 \cdot 6H_2O$	0.418				0.050		0.146			
6	$Na_2HPO_4 \cdot 12\ H_2O$	0.0450	0.006							0.012	
7	$(NH_4)_2\ SO_4$	0.0413					0.0113				0.030
8	Glucose	0.825									
	Total ions		1.129	0.095	0.165	0.050	0.0113	1.538	1.237	0.012	0.030
	Determined levels		1.126	0.095	0.165	0.050	-	1.141	1.120	0.010	-

Attempts to dissolve all the above compounds simultaneously in distilled-deionised water resulted in precipitation of calcium and magnesium carbonates. This was overcome by dissolving compounds 1, 2, 3, 6, 7, and 8 in about 800 ml distilled-deionised water; dissolving compounds 4 and 5 in about 100 ml distilled-deionised water; mixing the 2 solutions cautiously with stirring; and finally making up to 1 litre. The pH of the balanced saline was measured as pH 8.00 i.e. almost exactly the value determined for haemolymph.

2) A basic culture medium based on the balanced saline

Table 10: Full composition of the basic culture medium (BCM), with compounds grouped as for the preparation of stock solutions. (* indicates non-determined values.)

Group I	Compound	Weight/litre
	NaCl	1.812 g
	$CaCl_2 \cdot 2H_2O$	0.605 g
	$MgCl_2 \cdot 6 H_2O$	0.418 g
	$(NH_4)_2 SO_4$	0.041 g
Group II	$NaHCO_3$	1.500 g
	$KHCO_3$	0.243 g
	$Na_2HPO_4 \cdot 12H_2O$	0.006 g

Group III	**Compound**	**Weight/litre**
	Glucose	0.825 g
Group IV	L-Cysteic acid	1.93 mg
	L-Aspartic acid	6.94 mg
	L-Threonine	18.45 mg
	L-Serine	24.22 mg
(Koch-Light)	L-Glutamic acid	23.87 mg
	L- -Alanine	40.80 mg
(Koch-Light)	L-α- amino -n- butyric acid	2.82 mg
	L-Valine	26.14 mg
	L-Methionine	6.33 mg
(Koch-Light)	L-Isoleucine	8.15 mg
	L-Leucine	11.03 mg
	Glycine	9.58 mg
	L-Proline	5.50 mg
(Sigma)	Hydroxy-L-Proline	4.09 mg
	L-Tryptophan	17.50 mg*
	L-Arginine mono-HCl	31.16 mg*
	L-Ornithine mono-HCl	12.76 mg*
	L-Lysine mono-HCl	12.50 mg*
	L-Histidine mono-HCl	13.51 mg*
	L-Phenylalanine	10.00 mg*
Group V	L-Tyrosine	16.44 mg*
	L-Cystine	56.65 mg

Group VI		Compound	Weight/litre
		Aneurine-HCl	0.025 mg*
		Riboflavin	0.025 mg*
	(Koch-light)	Pyridoxine-HCl	0.0625 mg*
		Choline chloride	1.250 mg*
		Calcium pantothenate	0.025 mg*
		Meso-Inositol	0.125 mg*
		Nicotinamide	0.0625 mg*
		Nicotinic acid	1.000 mg*
		D-Biotin (Cryst.)	0.025 mg*
	(Sigma)	p-Aminobenzoic acid (K^+ salt)	0.125 mg*
Group VII		Folic acid	0.025 mg*
Group VIII		Vitamin B_{12}	1.000 mg*
Group IX		Calciferol	0.25 mg*
		Menadione (Vit. K.)	0.025 mg*
		Retinol acetate	0.25 mg*
		α-Tocopherol acetate	0.025 mg*
Group X		L-Ascorbic acid	0.0499 g*
		L-Cysteine-HCl	0.2599 g*
		L-Glutamine	0.1357 g*

Group XI	Compound	Weight/Litre
	Sodium Penicillin G.	20,000 units *
	Streptomycin sulphate	0.050 g
Group XII	0.05N NaOH as required to adjust pH to pH 8.00.	*

* indicates non-determined values.

All the inorganic chemicals were of Analar grade, and organic compounds were from B.D.H. unless otherwise stated. It was found most convenient to store the groups of compounds as follows:

Group I (inorganic salts less HCO_3^- and PO_4^{---}) was stored as a stock solution at x 10 concentration in distilled-deionised water. 100 ml aliquots of the stock solution were sterilised by candle filtration and stored in roller tubes at - 25°C.

Group II (HCO_3^- + PO_4^{---}) were stored as Group I.

Group III (Glucose) was stored as a stock solution at x 100 concentration in distilled-deionised water. 10 ml aliquots were sterilised by autoclaving, and stored in roller tubes at -25°C.

Group IV (soluble amino acids) were stored as a stock solution at x 100 concentration, prepared using distilled-deionised water warmed to 80°C. 10 ml aliquots were sterilised by candle filtration and stored in roller tubes at -25°C.

Group V (sparingly soluble amino acids) were stored as group IV preparing the stock solution in cold 0.1N HCl.

Group VI (Soluble vitamins) were stored as a stock solution at x 2,000 concentration in distilled-deionised water. The stock solution was sterilised by candle filtration and stored at -25°C. Some Biotin remained undissolved.

Group VII (Folic acid) was stored as a stock solution x 100 concentration in distilled-deionised water. The stock solution was sterilised by candle filtration and stored at -25°C.

Group VIII (Vitamin B_{12}) was stored as a stock solution X 1000 concentration in distilled-deionised water. The stock solution was sterilised by candle filtration and stored at -25°C.

Group IX (Alcohol-soluble vitamins) were stored as a stock solution at X 10,000 concentration in analar ethyl alcohol, at -25°C.

Group X (miscellaneous) were stored separately as solids at $-4^{o}C$.

Group XI (Antibiotics) were stored as a stock solution at X 100 concentration in distilled-deionised water. 10 ml aliquots were stored in roller tubes at $-25^{o}C$.

Group XII (Sodium hydroxide) was stored as an aqueous solution at room temperature.

Using the above scheme, 1 litre lots of BCM could be made up very easily and rapidly. The procedure for this was as follows. Deep frozen solutions except Group II and XII were then transferred to a 1 litre volumetric flask, washing the storage tubes thoroughly with sterile distilled-deionised water and adding the washings to the flask. The roller tubes and other vessels for storing stock solutions were themselves sterilised before use. The 100 ml aliquot of Group I solution and the 10 ml aliquots of Groups III, IV, V and XI solutions were taken first and then appropriate volumes of Groups VI, VII, VIII and IX solutions added by graduated pipettes. The Group X compounds were then weighed out as separate solids and dissolved directly in the flask. The volume of solution in the flask was then made up to around 800 ml and then the 100 ml aliquot of

Group II solution washed in. i.e. a similar procedure was used to that described for making up the balanced saline, except that here the bicarbonate fraction was added last. Diluting to 1 litre final volume at this stage gave a final pH of 7.1, because the HCl in the stock solutions had lowered the pH below that found in the balanced saline (see results 1.)). Thus, before diluting to 1 litre, Group XII solution was added to adjust the final pH to 8.00. Usually 6.5 to 7.0 ml were required. The addition of this alkali caused a temporary cloudiness in the solution which disappeared immediately on shaking. The complete medium BCM was stored deep-frozen or at $4^{o}C$ for several days. If deep-frozen for long periods it was found that the solids that separated out during freezing could not be easily re-dissolved. BCM was always made up using sterile distilled-deionised water, and finally sterilised by candle filtration immediately after making up.

Discussion

A fully defined protein-free and lipid-free medium has been developed based on the haemolymph analysis and on empirical addition of non-determined compounds. This should provide a suitable environment for at the least survival of larval Fasciola hepatica and addition of macromolecules, e.g. Dextrans; and lipids, e.g. Tween 80, to produce more complex media, is a future possibility.

Young sporocysts are probably highly adaptable animals, being little more than miracidia minus a layer of cells, as the change of environment from free-swimming in water, to living in snail tissue is extreme. It may be, therefore that young sporocysts will only require a simple medium like BCM containing merely an energy source, essential amino acids, and growth factors. Studies on the miracidium (Cheng, 1963) have shown that it carries considerable endogenous reserves of glycogen and lipid. It seemed therefore reasonable to attempt batch culture experiments using BCM, starting with miracidia or young sporocysts. Prospects for supplying a defined culture medium for rediae, which may need solid protein substrates, seem less hopeful.

It is difficult to speculate on the effects of gasing with a gas phase containing CO_2, on parasites living in BCM. As the measured pH of haemolymph is around 8.0, any lowering of the pH could have a detrimental effect. Such gas phases could be tried, however, together with the addition of solid $CaCO_3$ to the medium, to mimic the calcareous corpuscles found in mollusc tissues. Also later work (Appendix I) shows that the pO_2 of pulmonate haemolymph is very low, and a controlled gas phase may be necessary for successful cultures. One final consideration is that BCM will have a different tonicity to haemolymph due to the absence of protein. Thus in the development of more complex media work should be done to determine the depression of freezing point of BCM with and without addition of proteins or substitute macromolecules.

PART V The "*in vitro*" culture of sporocysts.

Introduction

There has been virtually no work to date on the "*in vitro*" culture of sporocysts. Barlow (1925), however, describes successful short term experiments in which the miracidia of *Fasciolopsis buski* shed their epidermal plate cells, changing into young sporocysts, when placed in a medium of "expressed snail juices". No quantitative criteria of development are given, and it appears that the cultures were non-sterile. It is encouraging, however, that Barlow also achieved metamorphosis of miracidia and some initial development of sporocysts in "filtered snail juice". He achieved metamorphosis both by treating hatched miracidia with "snail juice" and by allowing miracidia to penetrate snails and then dissecting out the young sporocysts. He did not consider this latter operation difficult, but it was not practical for the present study, being both time-consuming and presenting difficulties in keeping the sporocysts sterile.

The work of authors who have observed epidermal cell shedding (e.g. Barlow, 1925; Campbell and Todd, 1955; Targett and Robinson, 1964) suggest that "*in vitro*" culture of sporocyst is best attempted using sterile eggs and miracidia as the starting point. Thus it was decided here to develop techniques for the preparation of sterile

eggs and miracidia and to study the behaviour of miracidia, and development of young sporocysts in BCM. Difficulties in finding criteria for growth and development "in vitro" are fully discussed in this section, and the electron microscope is used to give a qualitative description of the development of cultured sporocysts.

Methods

1) Collection and incubation of sterile eggs, and procedures for obtaining sterile miracidia.

Infected sheep and cattle livers were obtained from the local slaughterhouse. Adult flukes were dissected from the bile ducts immediately after collection of the livers. This dissection was not performed under any semblence of sterile conditions, and the dissected flukes were stored in a crystallising dish in non-sterile distilled water at 4^{o}C. Dissected flukes were not used for egg collection beyond 24 hours after dissection from the liver. The flukes were rinsed thoroughly in non-sterile distilled water, removing all bile mucus, blood and tissue fragments from the dish. Damaged flukes were then picked out and discarded.

The following procedure was devised for egg collection. The procedure was performed in the open laboratory.on a bench previously

swabbed extensively with 70% alcohol. Normal sterile procedure was followed in the preparation of pipettes and in the transfer of materials between containers. Sterile distilled water was prepared by autoclaving at 15 p.s.i. for 20 minutes in screw-top vessels, and all glass and metal apparatus was sterilised by dry heat at 160°C for 3 hours initially, and by flaming and quenching in 70% alcohol as required during the procedure. Tissue paper was sterilised by dry heat in metal cans. Figure 7. shows the apparatus used.

Flukes were taken singly for egg collection. Each fluke was picked up with sterile forceps, and immersed in a bath of 70% alcohol for 3 seconds. During this time, the fluke was moved vigorously through the alcohol to rinse off mucus etc. The fluke was then removed from the bath and placed on a sheet of sterile tissue paper, and its anterior portion (Figure 7.a)) quickly severed with a sterile razor blade. This was transferred to a sterile 250 ml conical flask, containing about 100 ml sterile distilled water, and having a 2 inch diameter glass petri dish inner as a temporary lid. Up to 50 flukes were processed on each occasion, the anterior ends being pooled in the flask. During the procedure, the sterile water in the flask was changed after

every 10 flukes, shaking the suspension of anterior ends vigorously; decanting off the water; and replacing with fresh at least 3 times between each 10 fluke batch. This washing procedure was repeated at least 5 times when all the flukes had been processed. Shaking and changing the water was an aid to surface sterilisation of the anterior ends, and also removed any residual alcohol which might have otherwise accumulated in the flask, and penetrated to damage the eggs. Finally the pooled anterior ends were left suspended in about 50 ml sterile distilled water, and then quickly decanted into a sterile 100 ml vortex beaker fitted with a screw-top lid and integral homogeniser blades (MSE). Sterile distilled water was added to eliminate any air space and the suspension was then homogenised on the MSE bench homogeniser S25-120 at full speed for 20 seconds. The elimination of air space in the beaker was thought to prevent damage to and fragmentation of eggs by swirling air bubbles. These have been shown to be very powerful erosive agents in, for example, sea foam.

After homogenising, the beaker was allowed to stand for 15 minutes. During this time, the majority of the freed eggs settled to the bottom (Figure 7.b.). The upper supernatant, containing the majority of the waste shredded tissue, was then drawn off using a wide bore pasteur pipette. The eggs and remaining tissue debris were re-suspended by shaking the beaker, and the suspension decanted into a separating funnel of approximately 100 ml capacity (Figure 7.c.)).

Sterile distilled water was added to fill the separating funnel, and the eggs left to sediment for 20 minutes. During this sedimentation period, the layer of contaminating solids was occasionally stirred with a sterile glass rod to dislodge trapped eggs. After sedimentation, the eggs were carefully tapped off from the base of the funnel into a sterile 100 ml. conical flask (Figure 7.d.)) leaving the contaminating solids behind. At this stage, the addition of more sterile distilled water gave a clear suspension of sterile eggs, the only contaminants being small dense tissue fragments; especially detached suckers. These sediment with the eggs after homogenisation. These contaminants were removed by pasteur pipette. This operation was not difficult as the contaminants settled as a layer on top of the eggs (Figure 7.d.)) after re-suspension and sedimentation in the conical flask. Finally, the eggs were shaken and sedimented in 5 changes of sterile distilled water and then transferred to a fresh 100 ml conical flask, suspended in about 25 ml sterile distilled water.

It was found that considerable numbers of eggs could be collected from the discarded supernatants and contaminating fractions from the above procedure. These were pooled in evaporating dishes and non-sterile eggs collected by sedimentation for other work e.g. for infecting snails.

Using the sterile procedure, large numbers (estimated at thousands) of sterile eggs could be collected from a 50 fluke batch, in under 2 hours. The eggs were normally split into several batches e.g. 5 flasks of eggs from a 50 fluke batch.

Wilson (1965) gives detailed descriptions of factors affecting incubation, embryonation, and hatching of eggs. His routine method of incubation at 25°C for 12 days was followed here. The eggs were shielded from light during incubation by wrapping the conical flasks in aluminium foil, still allowing gaseous exchange through cotton wool plugs. Re-sterilisation of incubated eggs was performed on some occasions after 4 to 5 days incubation. At this stage of embryonation, exposure to light does not stimulate premature hatching, and the eggs were washed in fresh sterile distilled water using low speed centrifugation in capped tubes. Also, antibiotics were added to some sterile egg preparations at the same concentrations used in BCM. These concentrations are far lower than those thought by Wilson to prevent hatching (Wilson, personal communication, 1967). It is possible, however, that even low concentrations of antibiotics could have a deleterious effect on eggs. On several occasions eggs failed to embryonate and the real cause of this was never determined. It could have been either a.) damage during homogenisation. b.) effects of antibiotics or c.) contamination with 70% alcohol either during dissection

of the flukes or during the hatching procedure (see below). It was finally concluded that the routine procedure gave egg preparations of adequate sterility without the need for the use of antibiotics, or re-sterilisation measures. If incubated beyond 12 days, the sterile egg preparations remained in good condition and free from the fungal contaminants that often swamped non-sterile eggs left for long periods. The only time at which fungal contamination was observed was when a non-sterile (i.e. unfiltered) solution of antibiotics was added to preparations. This produced beautiful cultures of Streptomyces sp. in the ensuing experiments! Egg preparations were normally incubated immediately after collection, but were on occasions held for several weeks at $4^{o}C$ before incubation.

The hatching of eggs to provide sterile miracidia was induced by exposure to light: the mechanism is discussed by Wilson (1968a). This procedure, and the subsequent inoculation of cultures was performed inside a sterile glove-box. The latter was constructed from a portable fume cupboard, sealing all seams with an epoxy resin filler, and fitting a perspex front bored to take SLEE 7 inch ports and neoprene gloves. A U.V. sterilising lamp; tungsten filament lamp for general illumination; and a bench centrifuge were kept as

permanent installations inside the glove-box. The glove-box was also fitted with a front blind, to shield the lab. from U.V. light during sterilisation, and tubing and filters to allow the interior to be flushed with sterile air.

Before the introduction of any apparatus, the whole interior of the glove-box was sprayed with 70% alcohol from an aerosol, and left for 3 hours. Sterile air was then flushed through for 30 minutes, and during this time all the apparatus required for hatching-off eggs and setting-up cultures was quickly introduced through a port hole. The surfaces of all introduced apparatus were swabbed with 70% alcohol before introduction. It was during this procedure that alcohol vapour residual in the glove-box or from the swabbing may have penetrated to damage egg preparations causing the failure to hatch which was seen on some occasions. The apparatus introduced to the glove-box was as follows. 1) The sterile egg preparations, still in darkened flasks. 2) Sterile BCM in a 1 litre Buchner flask sealed with a silicone bung and tubing. 3) Metal cans containing sterile pasteur pipettes; sterile graduated pipettes of variable size according to the culture apparatus used; and sterile conical

centrifuge tubes with oxoid metal caps. 4) Sterile culture vessels (see below). 5) A foil bundle containing auto-claved pro-pipettes and bulbs for the pasteur pipettes. With all the apparatus introduced, and the flushing complete, the interior of the glove-box was further sterilised for 15 minutes using the U.V. lamp. The complete procedure gave very good sterile conditions inside the glove-box as shown by the sterility of dishes of nutrient media left exposed in the interior for test purposes. The media used for this were BCM, potato dextrose agar, and Difco nutrient agar.

Wilson (personal communication, 1968) has commented on the inhibition of hatching by concentrated salt solutions. It was felt here, however, that it would be much easier to suspend and hatch eggs directly into BCM than to allow them to hatch in water and then transfer the miracidia to the culture medium. Luckily it was found that BCM did not inhibit hatching. The foil was first stripped off the egg flask and then the egg suspension vigorously shaken and decanted into the centrifuge tubes. These were filled to the brim with sterile BCM, capped, and spun briefly on the centrifuge, merely taking it up to 2,000 r.p.m. and then switching off immediately. Using the centrifuge, the eggs were shaken and washed in 3 changes of sterile BCM and then left to stand for

hatching. After the initial exposure to light, about 5 minutes were available for centrifuging and washing the eggs. Hatching commenced after about 9 minutes, and the tubes were left to stand for 15 to 20 minutes to ensure hatching of all embryonated eggs. The very gentle centrifugation avoids packing down the eggs and damaging them. The procedure provided large numbers of miracidia swimming in BCM. They always congregated in the upper part of the tubes, nearest to the tungsten filament lamp, and could be withdrawn by pasteur pipette in a small volume of BCM for transfer to the culture vessels, completely free from egg shells.

Figure 7.

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2) Preliminary experiments towards culture in BCM.

Sterile miracidia were obtained as above, and transferred to solid watch-glasses containing sterile BCM, fitted with glass plates as lids. The behaviour of the miracidia, and cell-shedding and metamorphosis to sporocysts was closely observed with a binocular microscope in the open lab. Numerous cultures were set up, and many reduced to non-sterile status with the removal of sporocysts for examination at higher magnification. The cultures were maintained at room temperature in the open lab. for 48 hours. The success of these cultures and the usefulness of solid watch-glasses as culture vessels are discussed below (Results 2.)).

3) Attempts to find suitable criteria to assess the development of sporocysts in culture.

Many authors, (e.g. Bell and Smyth, 1958; Smyth (1956, 1959)) have stressed the importance of finding criteria to assess growth and development of parasites cultured "in vitro" and to distinguish this from mere survival. Young sporocysts of Fasciola hepatica present great problems in this respect as they are very small (i.e. around miracidial size), and their shape is continually changing due to body contractions (Barlow

1925). Added to this they are extremely difficult to manipulate due to surface mucus (see below). These factors made it impossible to develop any criteria based on physical or chemical measurements. Smyth (1956) suggests aceto-orcein squashes and subsequent mitosis counts as a good criterion of growth and development, and (1959) also describes qualitative schemes of criteria based on the development of gonads etc. The use of such schemes in the present study was greatly hampered by the lack of knowledge of how sporocysts grow and develop "in vivo".

Aceto-orcein squashes were tried on the germ cells of cultured sporocysts both with and without preliminary incubation in BCM containing colchicine (Smyth, 1956). The culture work described below shows that the development shown by sporocysts in BCM was not sufficient to devise a suitable scheme of qualitative criteria, especially as the small size of young sporocysts makes adequate observation impossible using the light microscope. Finally it was decided that an electron microscope study was the only way to assess any development of sporocysts in BCM.

4) Culture experiments, and techniques used for electron microscope studies.

Preliminary work was done to assess the usefulness of

various types of culture vessel. The vessels used were 1) solid watch-glasses with glass-plate covers. 2) 20 ml roller tubes. 3) 2 and 4 inch glass petri dishes. 4) 2 and 4 inch Carrel flasks fitted with silicone bungs. 5) assorted McCartney bottles. 6) assorted flat medicine bottles. 7) 4 inch pre-sterilised disposable plastic petri dishes. Attempts were made to fit gasing devices to numbers, 4) to 7) and, using mixtures of air and nitrogen with a flow-meter system (Rota-meters), to provide the cultures with a constant gas phase of low pO_2 (see Appendix I). These systems never worked satisfactorily due to unstable flow rates, and no gas phase apparatus was used in routine cultures with BCM. Flow-meters were not found to be a suitable means of producing known gas phases and it is probably best to order certified gas mixtures for such work. For ease of handling, convenient size, ease of observation of cultures, and for suitability of the surface for cell-shedding (see below), the vessels 7) above were chosen for routine culture work. The only disadvantage of these vessels was that condensation on the lids sometimes hampered observation.

Numerous culture experiments were run, adding sterile suspensions of miracidia to 25 ml volumes of sterile BCM in 4

inch disposable plastic petri dishes. This operation was always performed immediately after hatching and inside the sterile glove-box. Cultures were always maintained at 18°C in a cooled incubator (Gallenkamp IH 330), closely observing metamorphosis into sporocysts and thereafter examining the cultures at daily intervals. The results 4) below describe a typical culture with counts of metamorphosing sporocysts and mortality records. Detailed records such as this were not kept for all experiments as the limitations of BCM soon became apparent.

Sporocysts were removed from culture for fixation for electron microscope studies at known intervals of time from hatching. The time-consuming nature of electron microscope work caused this study to be rather incomplete, but the results obtained do give a general picture of the ultra-structure of sporocysts after 1, 3 and 7 days of culture in BCM. For comparison, a brief electron microscope study was made of sporocysts dissected from Lymnaea truncatula. These were obtained from experimentally infected snails, 8 days after penetration. In contrast to Barlow (1925) it was found to be almost impossible to dissect realistic numbers of younger sporocysts from snails for the electron microscope studies.

Sporocysts were left for 30 minutes after removal from culture to recover from manipulation and assume normal shapes and contraction

patterns. This and the fixation procedure were performed in solid watch-glasses at 4°C. Fixation was either by Zetterqvist's buffered osmium tetroxide (after Kay, 1965) or in 3% glutaraldehyde in pH 7.2 cacodylate buffer for 30 minutes followed by post-fixation in osmium for 4 hours. Fixation in Zetterqvist's buffered osmium tetroxide was for 2 to 4 hours. Fixed specimen were progressively dehydrated in ethyl alcohol, staining with 1% uranyl acetate for 30 minutes at both the 70% and 90% alcohol dehydration stages. Dehydrated speciments were transferred via propylene oxide to an araldite/epon mixture of the following composition: Epon 812 (Gurr), 25 parts; DDSA (HV964), 55parts; Araldite M (CY212), 15 par ts; dibutyl phthalate, 2 parts; BDMA accelerator, 3% by volume. The araldite,hardener and accelerator are all CIBA products. The mixture + specimens was polymerised in foil-lined 2 inch glass petri dish inners for 3 days at 60°C. Individual specimens were then isolated in small pieces of cured resin and trimmed to approximately 1 mm cubes. These small blocks were orientated for longitudinal or transverse sectioning and mounted on perspex rod with araldite general purpose adhesive. Sections were cut on the Huxley ultra-microtome and collected on grids coated with formvar and carbon. Sections on the grids were stained from 10 to 30 minutes with Reynold's lead citrate solution, and viewed in an AEI EM6B electron microscope, operating at 60 KV. Identical procedure was used for the sporocysts dissected from snails.

Cultures were tested for sterility before being used to provide specimens for electron microscope study. The liquid medium was first examined for fungi and for bacterial motility, and then inoculated to test media. Media used were potato dextrose agar and Bacto-Difco nutrient agar, incubated both at room temperature and at 37°C in the warm room. In addition to this, samples of BCM from the culture were incubated at room temperature and at 37°C.

Results

1) The procedures for preparing sterile eggs and miracidia were totally satisfactory and the occasional failure of eggs to hatch was attributed to a variety of reasons given in "Methods" above. In all cultures carried on beyond 24 hours and tested for sterility (see methods 4.)), sterility was proved. The speed and time of homogenisation is probably critical and probably affects the percentage of eggs that will hatch. This merits further work. BCM itself appeared to have no inhibitory effects on hatching.

2) Very variable results were obtained with short-term watch-glass cultures from the point of view of miracidia shedding epidermal cells. At best, the majority of miracidia shed cells after 1 to 1½ hours in BCM, and at worst only a very few miracidia had shed cells after up to 10 hours. Miracidia when hatched into BCM

were extremely active, swimming very fast, and often turning in tight circles. Some settled to the floor of the watch-glass cavity to shed cells, whereas others swam erratically through the medium while cells were being detached. Cell-shedding was very much as described by other author's (e.g. Barlow, 1925) and the epidermal cells appeared to be shed from the head end first i.e. simulated penetration. Viewed under high power magnification, some of the cells appeared to be actively swimming away, although when the process of shedding was complete, the majority of the young sporocysts still had a number of rounded-off epidermal cells stuck to them (see later plates).

After cell shedding, the young sporocysts tended to "round-off", becoming almost spherical in shape. The eye-spots remained prominent, and flame cell activity was easily observed. The young sporocysts showed convulsive twitching movements, but appeared to be firmly stuck to the glass by surface mucus. This was confirmed by the extreme difficulty in manipulating sporocysts using capillary pipettes. The specimens often stuck to the inside of the pipette and were impossible to dislodge.

It should be noted that in most cultures a very few miracidia remained unchanged and actively swimming after up to 24 hours in BCM, usually dying or continuing to swim feebly before the cultures were abandoned (see Results 4.) below). This preliminary work at

least showed that metamorphosis is obtainable in BCM, and raised interesting questions as to the mechanism and stimulus for cell-shedding. It was encouraging that the antibiotics and 0.01% ethanol in BCM had no short-term deleterious effects on miracidia or young sporocysts.

3) Attempts to measure and weigh sporocysts were all complete failures. The aceto-orcein squashes likewise produced no worthwhile results. Germ cell nuclei were located, but no mitoses were identified with certainty, even after extensive incubation with colchicine. It was thus decided to use the electron microscope to give qualitative descriptions of development in culture.

4) Preliminary work showed that miracidia would shed epidermal cells in BCM on both glass and plastic surfaces, the process starting from 1 to 5 hours after hatching. No data are available to assess the relative merits of different surfaces, but plastic surfaces were judged subjectively to be better than glass, and subsequent cultures in 4 inch disposable plastic petri dishes were generally very reliable, with metamorphosis often starting under 1 hour from hatching. The equipment for controlling gas phases never worked satisfactorily and was eventually discarded. It was apparent that

attachment to the plastic surface was not essential for metamorphosis to start, but was essential for continued survival of the young sporocysts. Unchanged miracidia swimming feebly in the medium and detached partially metamorphosed individuals invariably died, and dead individuals of all stages became detached from the plastic surface. Table 11. gives the counts of various types of individual seen in a set of 3 typical cultures up to 96 hours after hatching. Figure 8. explains the "type" system used. The numbers given are the total observed in 3 fields of view for each culture, viewed with a dissecting microscope and counted with a hand tally.

Table 11. Miracidium/sporocyst "types" observed in cultures 1, 2 and 3 in BCM (pH 8.00), counted at 24, 48, 72 and 96 hours after hatching. Percentages are given to 1 decimal place

		Types (see Figure 8.)							
Time	Culture	A	B	C	D	E	F	G	Total
24 hours	Culture 1	7	10	170	41	16	15	0	259
	Culture 2	13	9	385	75	38	17	0	537
	Culture 3	2	3	193	21	9	4	0	232
Total		22	22	748	137	63	36	0	1028
%		2.1	2.1	72.7	13.3	6.1	3.5	0	

Time		A	B	C	D	E	F	G	Total
48 hours	Culture 1	1	5	125	39	19	1	69	259
	Culture 2	3	7	244	52	45	2	83	436
	Culture 3	0	2	123	20	10	0	111	266
	Total	4	14	492	111	74	3	263	961
	%	0.4	1.5	51.2	11.6	7.7	0.3	27.4	
72 hours	Culture 1	0	0	6	36	22	1	198	263
	Culture 2	0	0	10	48	40	1	325	424
	Culture 3	0	0	2	16	10	0	163	191
	Total	0	0	18	100	72	2	686	878
	%	0	0	2.1	11.4	8.2	0.2	78.1	
96 hours	Culture 1	0	0	0	34	20	0	225	279
	Culture 2	0	0	3	50	36	0	341	430
	Culture 3	0	0	1	12	13	0	175	201
	Total	0	0	4	96	69	0	741	910
	%	0	0	0.4	10.5	7.6	0	81.4	

After 96 hours there was virtually no mortality among Types D and E which were considered to be successfully metamorphosed sporocysts,

showing strong attachment to the plastic surface, continuous body movement, and having rapidly beating flame cells. The percentage of Types D and E can be seen to remain constant at around 20% after metamorphosis, and this was the general rule for all cultures. The epidermal cells ramaining attached to Types D and E were merely stuck to surface mucus and were easily detached by stirring the medium around the sporocysts or by the pipetting procedures. Plate 11 shows typical Type D sporocysts, 24 hours after hatching. Table 11 shows the tremendous increase in Type G dead individuals, which have obviously failed to complete metamorphosis. Successfully established sporocysts could always be distinguished from Type C individuals or dead sporocysts simply by rocking the culture vessel. The latter would move in the displaced medium, whereas successful sporocysts always showed strong attachment to the plastic surface. This was a great help when removing specimens from culture for compound microscope studies, or fixation for electron microscope studies. It deserves mention that attached sporocysts and the process of metamorphosis could be best observed using an inverted compound microscope of the type used for observing cell and tissue cultures in flat medicine bottles. This allowed body movements and flame-cell activity to be studied without disturbing the cultures, and also showed that attachment to the plastic surface, during the early stages of metamorphosis, was always by the apical papilla of the miracidium, as in snail penetration.

Table 11. shows that, as in results 1.) above, significant numbers of miracidia are not stimulated to shed epidermal cells at all, even up to 48 hours after hatching (and beyond in a few cases). These individuals undoubtedly end up as Types F and G. The large numbers of Type F recorded at the 24 hour stage were probably miracidia damaged during the hatching process, and these later degenerate to Type G.

The general conclusion from the culture work was that around 20% of the miracidia hatched into sterile BCM survived as young sporocysts throughout the cultures, although up to 70% achieved a partial metamorphosis (Type C) during the first 48 hours. Sporocysts were kept for up to 9 days with negligible mortality, but at this stage the cultures were discontinued since no interest was held in abnormally long survival times with no apparent growth and development ("in vivo" sporocysts are producing rediae after 9 days). No noticeable growth or development occurred during the cultures, and most of the successfully established sporocysts still retained a distinct apical papilla. The only change observable with the light microscope was disruption of the eye-spot pigment. This was often seen to be pushed around inside the sporocysts by body contractions. Table 11. shows the different densities of cultures resulting from the addition of empirical amounts of miracidial suspension. It

was estimated that most cultures contained around 1000 miracidia initially.

The electron microscope work, however, made up a little for the disappointing results above. The extreme stickiness of the sporocysts made manipulation before fixation very difficult. Types D and E were used exclusively for the electron microscope studies, and any remaining epidermal cells were usually stripped off during the pipetting procedure. Eventually, satisfactory pictures were obtained of the ultrastructure of sporocysts after 24, 90 and 168 hours in culture. Zetterqvist's buffered osmium tetroxide was judged to be by far the better of the two fixatives used. Plates 12 to 30 illustrate the work. The observations that follow do not provide a very complete picture, but, in showing the inadequacy of BCM for supporting growth and development comparable to that "in vivo", they do shed some interesting light on metamorphosis and early development. A discussion on cell-shedding is included at the end of this part, with further comparisons of the ultrastructure of sporocysts and miracidia (Wilson 1969a).

Wilson (1969a) gives a very full description of the ultrastructure of the miracidium, and the changes occurring during metamorphosis

"in vitro" are best understood with reference to this work: particularly the structure of the body wall. Plates 12. and 13. show the structure of the miracidial body wall, and Plate 30. gives a further comparison with the body wall of a redia and a cultured sporocyst. Plate 12. shows the typical ciliated epidermis and its inclusions; the thin cytoplasmic layer; and deeper tissues including a vesiculated cell (Wilson, 1969a). Plate 13. shows the outer layers in greater detail including a lysosome-like body in the epidermis, the significance of which will be discussed later.

Electron microscope studies of the sporocysts dissected from snails also showed many interesting features, and indicated the type of developments to look for in the cultured forms: remembering that the "in vivo" sporocysts were 9 days old. Plates 14. to 17. illustrate the work and the structure of the body wall is the most interesting feature. There is a superficial resemblence to the redial body wall (Plate 30.) in that the body surface is corrugated, and the corrugations appear as micro-villi in section. Plate 17. shows these corrugations in what is almost a surface view. The body wall itself contains a variety of inclusions such as ribosomes and various vacuoles (Plate 15) and mitochondria (Plate 16) but has no nuclei. It has a very distinct basement membrane and Plate 16 shows one of the very rare breaks in this, allowing communication

with the deeper tissues. Within the body wall it is sometimes difficult to distinguish between the tissues of the sporocyst and those of the developing rediae, but muscle blocks and various types of cell can clearly be seen outside the developing redial body wall (Plate 14.). Some of the large cells contain dark vesicles, but with sporocysts of this age, it cannot be said that these are definitely persistent vesiculated cells from the miracidium (Wilson, 1969a). It is probable, however, that many of the cells are derived from the vesiculated cells and nutritive cells described by Wilson (1969a).

The observations on "in vitro" cultured sporocysts showed that two main changes occurred during the first 24 hours in culture, associated with the metamorphosis from miracidium to sporocyst. The first change concerns the development of the sporocyst body wall. Plates 18. to 21. illustrate the body wall of sporocysts after 24 hours in culture. Comparison with the body wall of the miracidium shows that the ciliated epidermal cells have been completely shed (this is readily observable with the light microscope) and that a new body wall has been formed, presumably from the thin cytoplasmic layer beneath the epidermis of the miracidium. The most striking aspect of this new body wall is its variability, and Plates 20. and 21. show the complex surface projections that can develop, in contrast to the smooth appearance of some parts (Plate 18,),

and the development of small micro-villi (corrugations ?) in others (Plate 19.). The new body wall also varied greatly in thickness being generally very thick towards the posterior end of the sporocyst, and thinner towards the anterior near the remains of the apical papilla. The extreme surface variation suggests that the young sporocysts were in an unsuitable physical environment, and their strong attachment to the plastic vessels may account for some of the strange body wall proliferations. It is interesting that after only 24 hours in culture metamorphosis has taken place and a new body wall has been formed resembling in its main features that of the sporocysts dissected from snails i.e. 1.) It is non-nucleate. 2) It has a basement membrane. 3) It contains similar inclusions. 4) In a few places it appears to be developing surface corrugations. The basement membrane is not as distinct as that seen in the "in vivo" sporocysts, but it does appear to be continuous. Plates 20. and 21. show that the dark vesicles are found in the new body wall. This fact, and the observation that miracidial-type vesiculated cells were almost completely absent from the cultured sporocysts, suggests that the vesiculated cells were involved in the production of the new body wall. Indeed in the short time elapsed i.e. 24 hours, it seems unlikely that this new body wall is the result of any synthesis of new material. It is almost certainly the result of a re-distribution

of materials allowing proliferation of the thin cytoplasmic layer of the miracidium, and it seems likely that a significant amount of this material derives from the vesiculated cells. Without any precise knowledge of the structure and function of the vesiculated cells, it is difficult to comment on their apparent absence in young sporocysts cultured "in vitro" and their positive identification in 9 day old "in vivo" sporocysts. However, the possible mechanisms for the re-distribution of material in metamorphosis are discussed later.

The second main change seen after 24 hours in culture is a massive "reorganisation" of internal tissues, particularly towards the posterior end of the sporocyst and around the brain and eye-spots. In some areas of "reorganisation" the picture appears highly chaotic with cellular inclusions found either free or enclosed in large membrane-bound vesicles. The latter were seen containing mitochondria, dense vesicles, golgi, and pieces of desmosome. Plates 22. and 23. show typical areas of "reorganisation", Plate 23. showing a huge "packet" of mitochondria and a strange fibrous structure of unknown origin. It seems likely that this breakdown of miracidial tissue has been achieved by lysosome activity and that these membrane-bound bodies are phagosomes which are mopping-up cell-debris for possible re-distribution elsewhere. It is impossible to say, without

extensive serial sectioning, to what extent "reorganisation" occurs in different parts of the young sporocyst.

In all the "in vitro" cultured sporocysts examined at 24 hours, the brain and nervous tissue appeared highly disrupted, together with the cells associated with the eye-spots. In some specimens, brain tissue was identified near the centre of the body in the germ cell region. This was undoubtedly because the breakdown and "reorganisation" processes allowed a mixing-up of the sporocysts internal tissues, assisted by body-contractions. Thus in cultured sporocysts it was possible to find such things as eye-spot pigment remains virtually anywhere in the body and intact flame cells displaced and lying in different planes to those seen in the miracidium (Wilson, 1967, 1969b). This internal mixing was also seen on numerous occasions when observing body-contractions and flame cell activity with the light microscope.

Apart from the absence of vesiculated cells and the regions of internal "reorganisation" the internal tissues of the "in vitro" cultured sporocysts at 24 hours were virtually the same as in the miracidium (Wilson, 1969a). The muscle system, nutritive cells, excretory system, and germ cells appeared unchanged, although nucleolus

formation - which could be a pointer to synthetic activity - was frequently observed in the germ cells (Plate 24.). The excretory system was virtually as seen in the miracidium (Wilson, 1969b), and some interesting pictures of flame cells were obtained. Plate 25. shows part of a flame cell in transverse section and it is interesting to note the differential staining of the background material inside and outside the barrel. The background matrix outside the barrel is of fine threads (probably soluble proteins precipitated during fixation) while that inside the barrel is clear. This effect was noticed in several different preparations and could be evidence for ultra-filtration: with macromolecules being retained outside the barrel. Wilson (1969b) discusses the functions of the flame cell system suggesting possible sites of filtration and analogy with glomerular filtration in the mammalian kidney.

Another structure remaining virtually unchanged from the miracidium was the apical papilla. However on <u>one</u> preparation from a 24 hour cultured sporocyst it appeared that the accessory gland cells (Wilson, 1969a) had lost their endoplasmic reticular contents suggesting that the miracidium had undergone a simulated penetration during its metamorphosis in BCM.

In the older cultured sporocysts (90 and 168 hours) there was

a disappointing lack of further development. The new body wall and areas of "reorganisation" (Plates 27. and 28.) remained roughly the same as at the 24 hour stage although in some specimens the basement membrane appeared to have been strengthened, having a corrugated appearance. Also, aggregations of dense vesicles, phagosomes, and remnants of reorganising cells were found actually in the new body wall: perhaps forced there by prolonged internal mixing. Germ cells, nutritive cells, and flame cells, showed no changes from the 24 hour stage. Indicative of the overall lack of growth and further development was the retention of the apical papilla, and the fact the eye-spot pigment material was hardly any further dispersed than at the 24 hour stage (Plate 29.).

Thus the general result of the electron microscope studies was to demonstrate the metamorphosis achieved in BCM and the formation of the sporocyst body wall, with "reorganisation" of internal tissues. While the new body wall resembled that of "in vivo" sporocysts in its basic structure, the internal "reorganisation" was difficult to interpret as leading to the development of a simple sac-like organism producing rediae, - particularly as the germ cells showed no division. This, and the inability of the new body wall to develop regular surface corrugations suggests that there must be doubts as to the normality of the metamorphosis achieved in BCM, and whether it corresponds to that "in vivo".

Discussion

The work with BCM is quite encouraging as both metamorphosis and continued survival of young sporocysts were achieved. Although only a minority of the miracidia completed metamorphosis in BCM, and the developments beyond 24 hours in culture were negligible, it is interesting to analyse the results more closely, particularly with respect to the processes involved in metamorphosis. Apart from the limitations mentioned above, and the presence of free-swimming miracidia up to as much as 48 hours after hatching, it seems that at least some of the changes associated with penetration of the snail did take place in the metamorphosis achieved "in vitro". Many of these changes are very imperfectly known, but cell-shedding and eye-spot disruption (indicative of internal "reorganisation") have been observed by many authors (e.g. Barlow, 1925; Campbell and Todd, 1955; Targett and Robinson, 1964).

The mechanism of cell-shedding has never been satisfactorily explained, but the present study and work on the ultra-structure of the miracidium (Wilson, 1969a) suggest solutions to the problem. It appears that all tissues external to the thin cytoplasmic layer (Plates 12. and 13.) are shed as discreet rounded-off cells, following dissociation of the peripheral desmosome by which the epidermal cells

are attached. This dissociation could be the result of some activity in the underlying thin cytoplasmic layer. Wilson (personal communication, 1968) has shown that various chemical agents will disrupt the peripheral desmosome and strip off the epidermal cells "in vitro". These include various enzyme treatments - particularly tryptic enzymes - and a variety of basic substances including basic dyes such as toluidine blue and alcian blue, and basic proteins such as histones and protamines. Many of these preparations killed the miracidia while causing the epidermal cells to strip off. The evidence suggests that the peripheral desmosome is a mucoprotein.complex and that enzyme treatments will attack the protein portion and certain basic substances disrupt the "muco -" portion. The latter is almost certainly due to selective binding of the acidic groups on the mucopolysaccharide branches of the mucoprotein complex. Pearse (1960) describes this property of basic dyes, and it appears that selective binding of the mucopolysaccharide acidic groups makes the whole complex more hydrophobic, causing shrinkage. This could account for the pulling-apart (dissociation) of the desmosome.

The above work, although interesting in showing the probable chemical nature of the peripheral desmosome, has little direct relevance to cell-shedding during natural metamorphosis. This is shown by the fact that cell-shedding has now been achieved "in vitro" in a medium

containing no enzymic or basic agents comparable to those mentioned above. Indeed cell-shedding can sometimes be observed when miracidia in water alone are trapped in lens tissue on a microscope slide. This suggests that there are no exogenous chemical agents, e.g. snail exudates, involved in the process of cell-shedding during natural metamorphosis. The very fact that cellsgare shed from the anterior end first suggests that an internal mechanism is involved and that the whole process is under fairly strict control. This shedding of cells progressively from the anterior end is not seen when chemical agents are used to strip off the cells. More observations are needed to show what actually happens to the desmosome as the cells peel off and electron microscope studies on specimens fixed during metamorphosis in BCM could clarify the situation.

It is interesting to speculate on the possible nature of the internal mechanisms initiating and controlling cell-shedding and the other aspects of metamorphosis. No receptor organs have been proved to exist in the miracidium but Wilson (personal communication, 1968) has shown that structures do exist which could have a sensory function, and it seems reasonable to postulate that the miracidium has adequate sensory apparatus to recognise the right conditions for penetration and metamorphosis. Under natural conditions then, sense organs could inform the brain of the location of a suitable

host, and penetration, cell-shedding, and internal "reorganisation" are thus likely to be initiated and controlled by the brain. This could be directly by nerve fibres, by slow diffussion of neurotransmitter substances, or by a combination of both. This activity of the nervous system could then release substances to cause controlled rupture of lysosomes in the epidermis and the tissues about to start "reorganisation". There is at present no definite proof of the presence of lysosomes in miracidial tissues, but the appearance of the areas of "reorganisation" (Plates 22. and 23.) and the presence of membrane-bound bodies in the epidermis do suggest that lysosomal activity is involved. Certainly the controlled rupture of lysosomes could bring about both cell-shedding - by enzymic disruption of the peripheral desmosome - and cellular "reorganisation", by for examples destruction of the vesiculated cells releasing materials to build the new body wall.

Further electron microscope studies are needed on sporocysts "in vivo" at known intervals after penetration. This would show if internal "reorganisation" follows the same course "in vivo" as in "in vitro". It is, however, extremely difficult to remove very young sporocysts from snails, as they become lodged in dense tissue and are easily damaged during dissection. A possible method of study would be to expose snails to multiple infaction andtthen,

after anaesthetisation, amputate tentacles for fixation and electron microscope studies. Sporocysts from miracidia that penetrate the tentacles are easily observed in the living snail with a dissecting microscope.

In assessing the usefulness of BCM, it does seem that it is a good basic medium from which other media could be developed. Here at least it supported miracidia through metamorphosis and survival of sporocysts for up to 9 days without any apparent osmotic stress. It is interesting to consider why no growth and development occurred in BCM after the 24 hour stage, and why only a minority of the miracidia achieved anything resembling a successful metamorphosis. In the latter case it is best to regard metamorphosis as being cell-shedding plus a complete internal "reorganisation" and thus the results achieved with BCM are really only partial metamorphoses.

First, the inability of many miracidia to achieve even this partial metamorphosis or to attach to the culture vessel, and the strange proliferations of the body surface of successfully attached sporocysts, suggest that the physical environment provided by the present cultures is far from ideal. It may be that the miracidia do require a solid substrate to penetrate for normal metamorphosis. Thus cultures using gels and precipitated protein slopes, perhaps with BCM as an

overlay, are future possibilities. While considering the physical conditions, it is also possible that the pO_2 of the culture medium is a critical factor. Appendix I shows the very low pO_2 of a pulmonate haemolymph and a controlled gas phase may be essential for successful cultures. It is improbable that extra buffering systems are needed to control pH, but a gas phase containing a known level of CO_2 and the addition of solid $CaCO_3$ to the cultures could be used as a bicarbonate buffering system. This would probably be difficult to arrange at pH 8.0 but it would provide a better simulation of conditions in the snail.

Second, BCM is almost certainly lacking in some vital chemical constituents. Apart from the lack of protein, which could not be added other than as pure preparations of known molecular weight, without rejecting the idea of a fully defined medium, the most obvious substances missing are lipids. Cheng (1963) mentionss the possible importance of lipids, and reviews previous work. Although miracidia carry reserves of lipids it appears that sporocysts may need a source of exogenous lipids. The relationships between the lipids of the host snail tissues and those of the sporocysts have been studied by Ginecinskij (1960) and Cheng and Snyder (1962a, 1962b). However the utilisation of exogenous lipids and the possible methods of transport across the sporocyst body wall are very imperfectly understood. It seems that the assumption (Part IV) that miracidia

carry enough lipid reserves for metamorphosis and early development, is wrong and that BCM will have to be supplemented with lipids for future work. There are great difficulties in incorporating lipid components into an aqueous culture medium, but future work should be done to see if the addition of fairly simple fatty acids could supply the sporocysts requirements. If the idea of a fully defined culture medium has to be eventually rejected, lipids could be added in combination with protein.

It is also possible that solid glycogen would be a useful addition source of carbohydrate in a culture medium. Cheng (1963) suggests that sporocysts may secrete a glycolytic enzyme to break down glycogen in the host tissues. It could be that solid glycogen would provide cultured sporocysts with an energy source which they could regulate themselves, and that this would be preferable to the present fixed level of glucose. It is not possible to speculate about any other specific chemical inadequacies of BCM. It is possible that trace cations and growth factors are also missing. The very full amino acid complement in BCM should be sufficient for an organism taking in nutrients through its body surface, but, as mentioned above, proteins may be necessary as part of the physical environment rather than as a source of amino acids.

It appears that much work will have to be done both to supplement and modify BCM and to improve culture apparatus. A continuous flow

apparatus with a constant gas phase is an interesting possibility. In the present study it appears that the sporocysts may not be taking any nutrients from BCM. Certainly all the changes involved in the partial metamorphosis could have been accomplished simply by redistributing material and using endogenous reserves of lipids and glycogen. Another essential piece of future work is a study of the growth and development of sporocysts "in vivo" as a first step towards the development of adequate criteria for future culture work.

PART V

Figure 7 Apparatus used in the collection and preparation of sterile eggs.

a) The fluke is cut along XX to isolate the anterior portion containing the uterus.

b) The layers of contaminants after homogenisation.

1. Main tissue fragments.
2. Cloudy supernatant.
3. Suckers and small heavy tissue fragments.
4. Eggs.

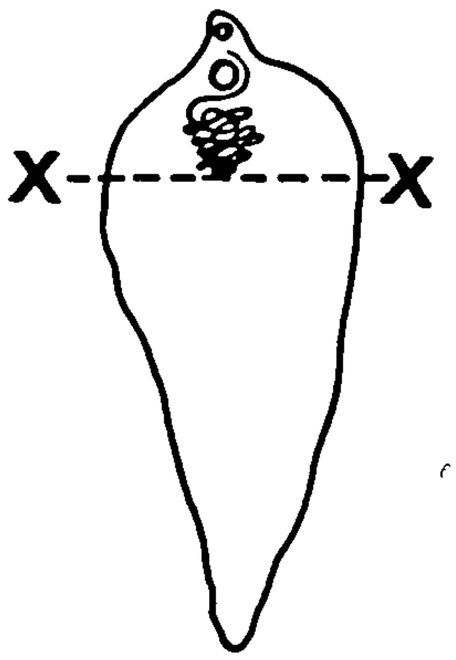
c) The use of a separating funnel to remove contaminants.

1. Cloudy supernatant.
2. Suckers and small heavy tissue fragments.
3. Eggs.
4. Foil to keep outlet sterile.

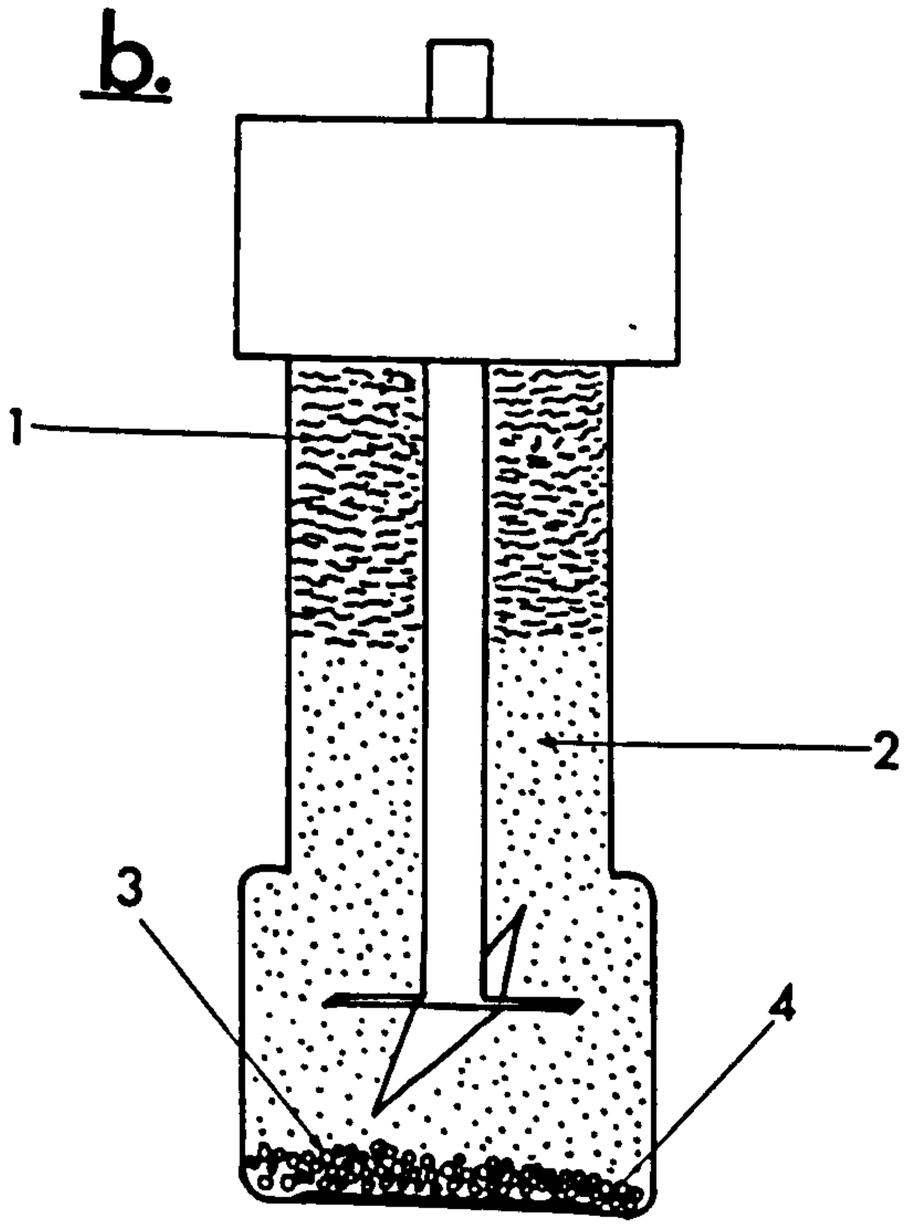
d) The final washing of eggs in a conical flask.

1. Clear supernatant.
2. Few remaining suckers and small heavy tissue fragments (removed by pipette).
3. Eggs.

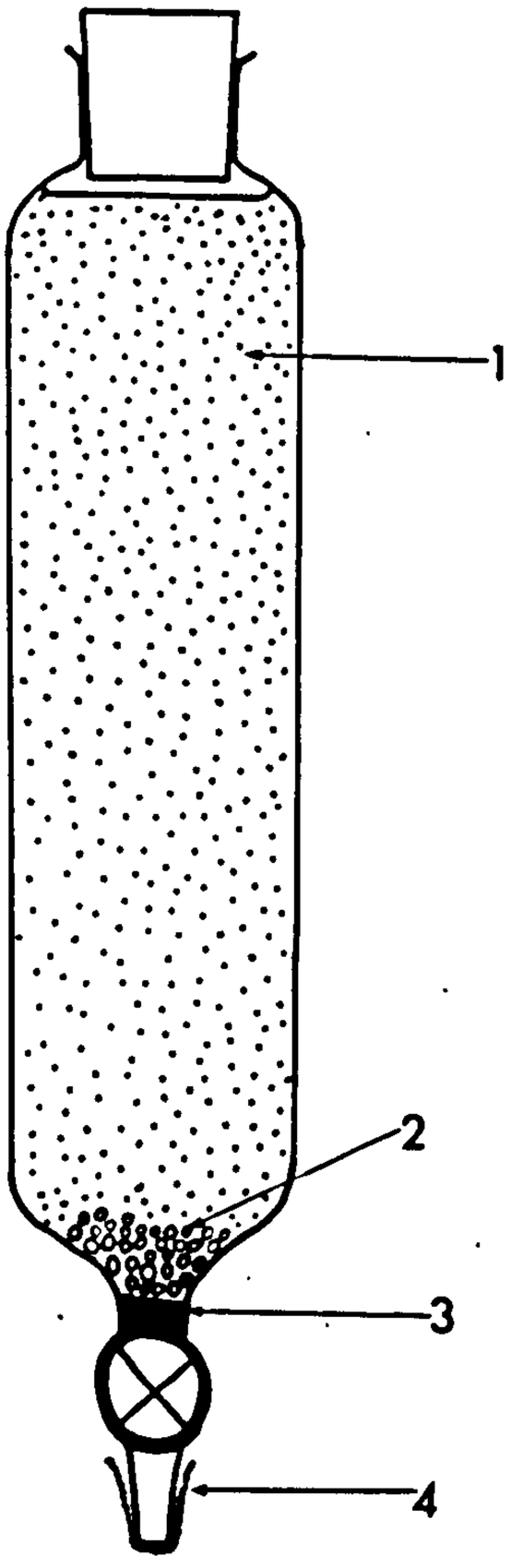
a.



b.



c.



d.

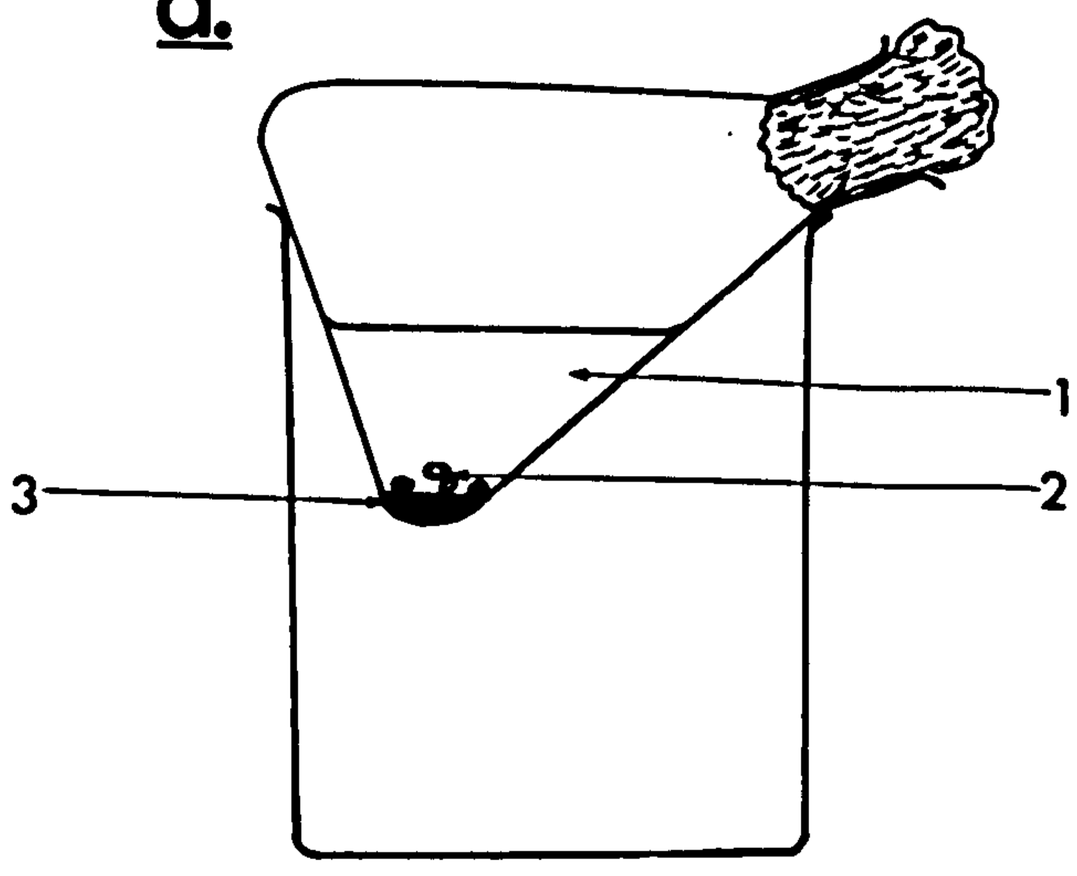


FIGURE 7

Figure 8 Miracidium and sporocyst types counted in Table 11.

Type A. Normal free-swimming miracidium.

Type B. Feebly swimming miracidium with cilia beating slow enough for easy observation.

Type C. Partially metamorphosed individual; with epidermal cells bulging but not detached; attached to the culture vessel by the apical papilla only; or free in suspension.

Type D. More completely metamorphosed individual, attached to the culture vessel along the whole length of the body, but with shed cells still superficially stuck to surface.mucus.

Type E. As type D. but with few or no cells remaining stuck on.

Type F. Stationary, dead miracidium which has failed to start metamorphosis; internal tissues broken down.

Type G. Dead, mishapen cytolysed individuals.

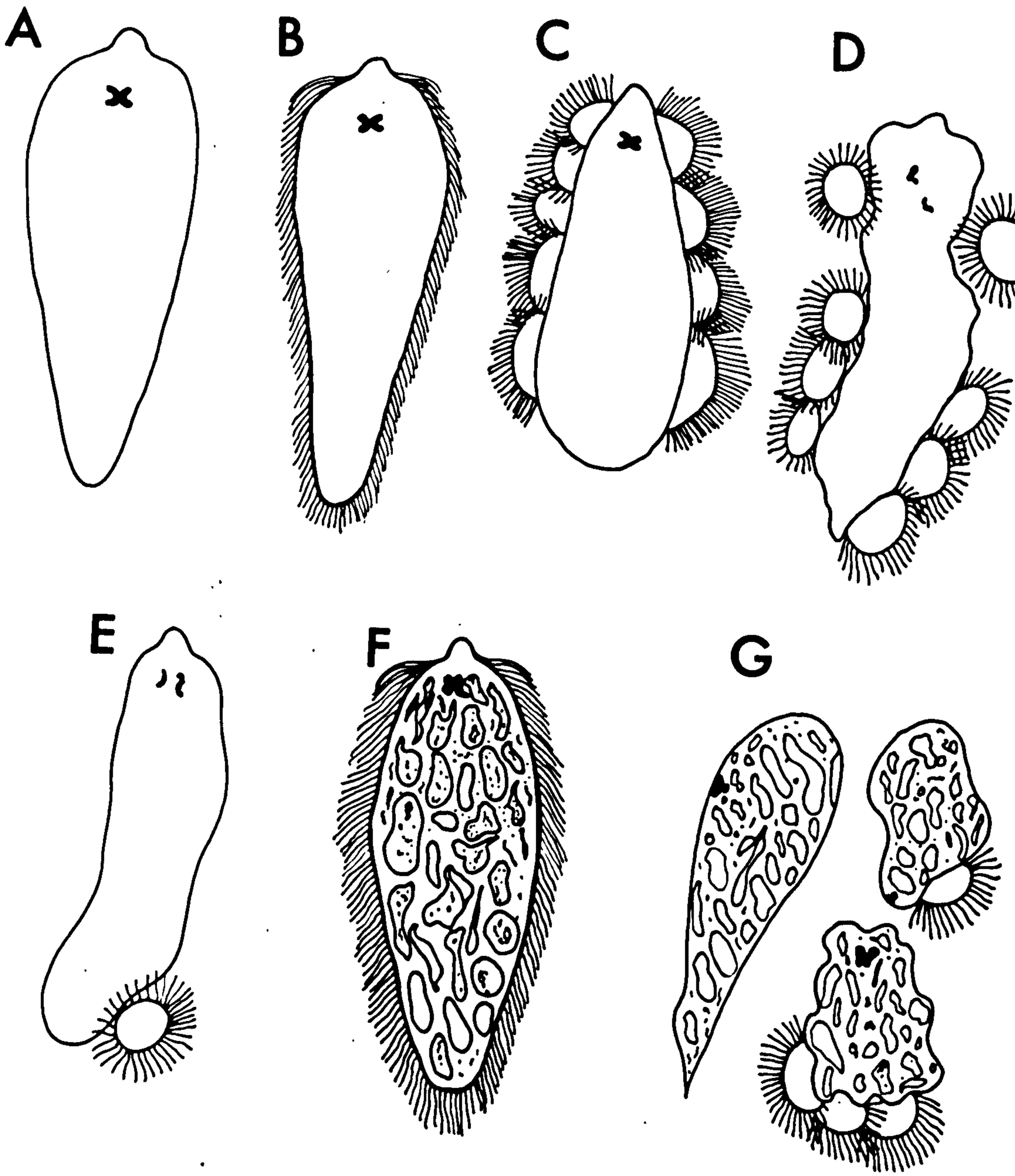
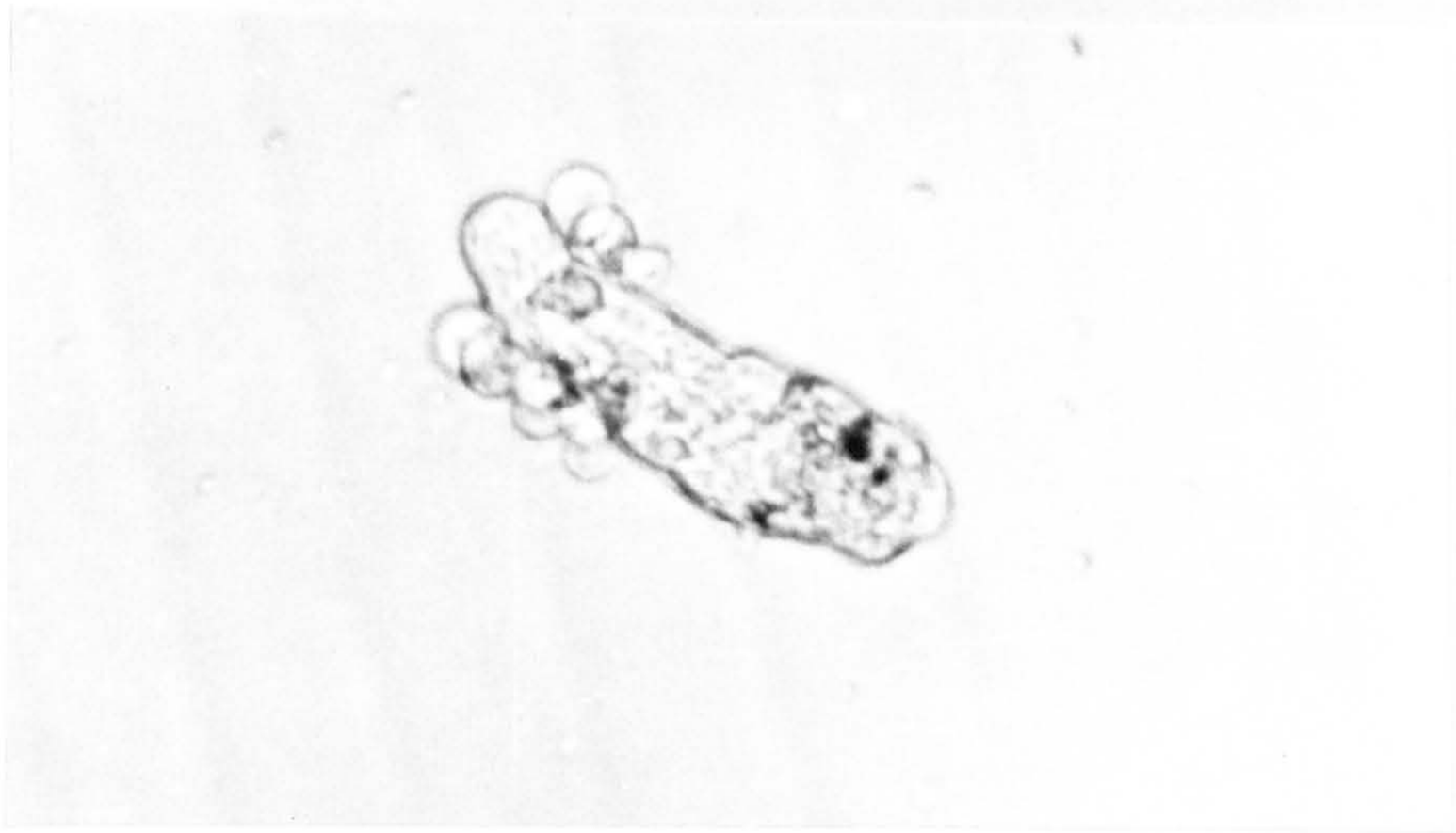


FIGURE 8

Plate 11

a) and b): Type D sporocysts in culture, 24 hours after hatching in BCM.

a)



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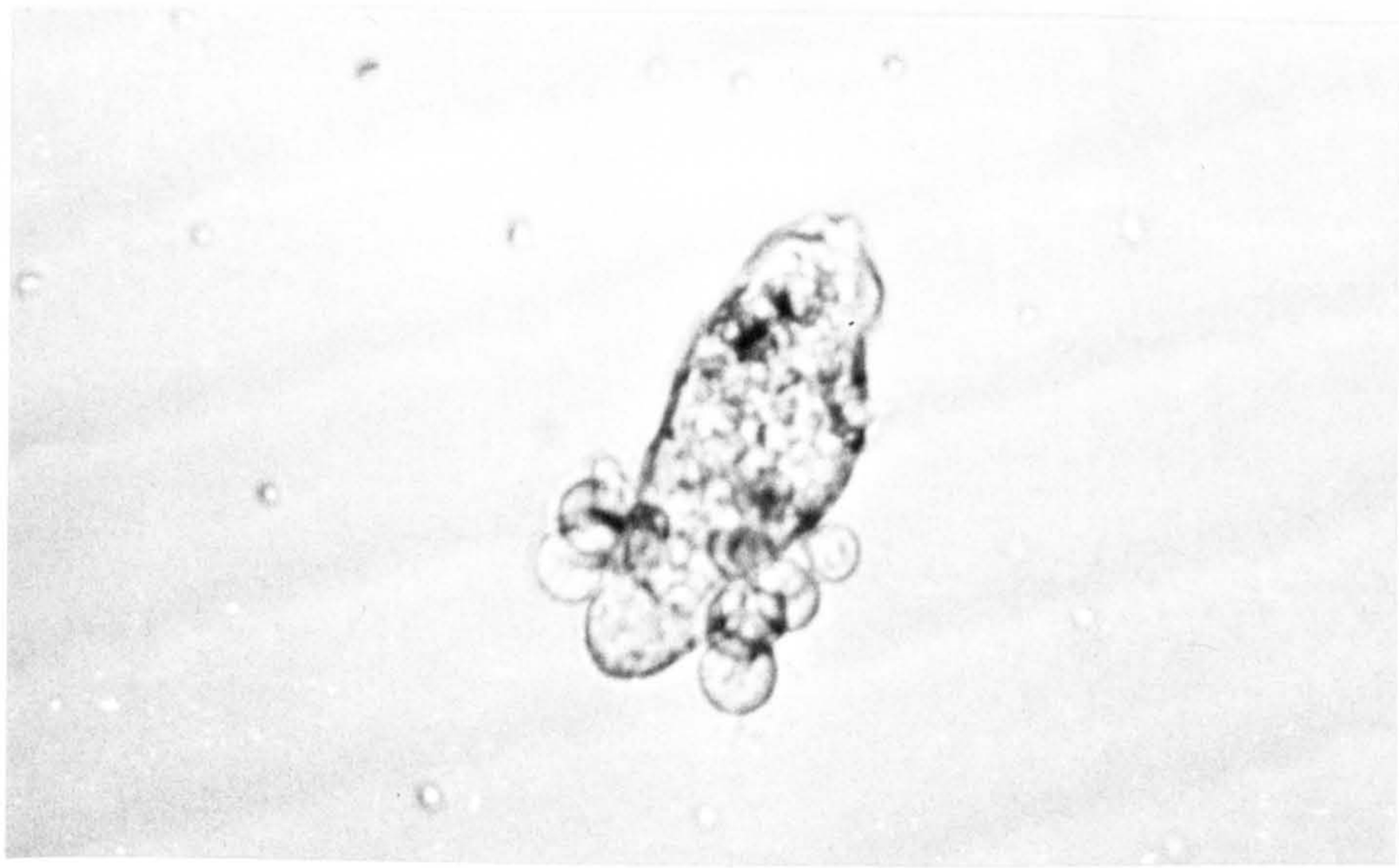


PLATE 11

Plate 12. Transverse section of a miracidium through the germ cell region showing the general structure of the body wall.
(From an electron micrograph donated by R.A. Wilson, 1968)

C - Cilium.

CE - part of an epidermal cell.

CM - circular muscle.

GC - part of a germ cell.

LM - longitudinal muscle.

M - mitochondria.

P - part of a protonephridial tubule.

PCP - parts of parenchyma cells.

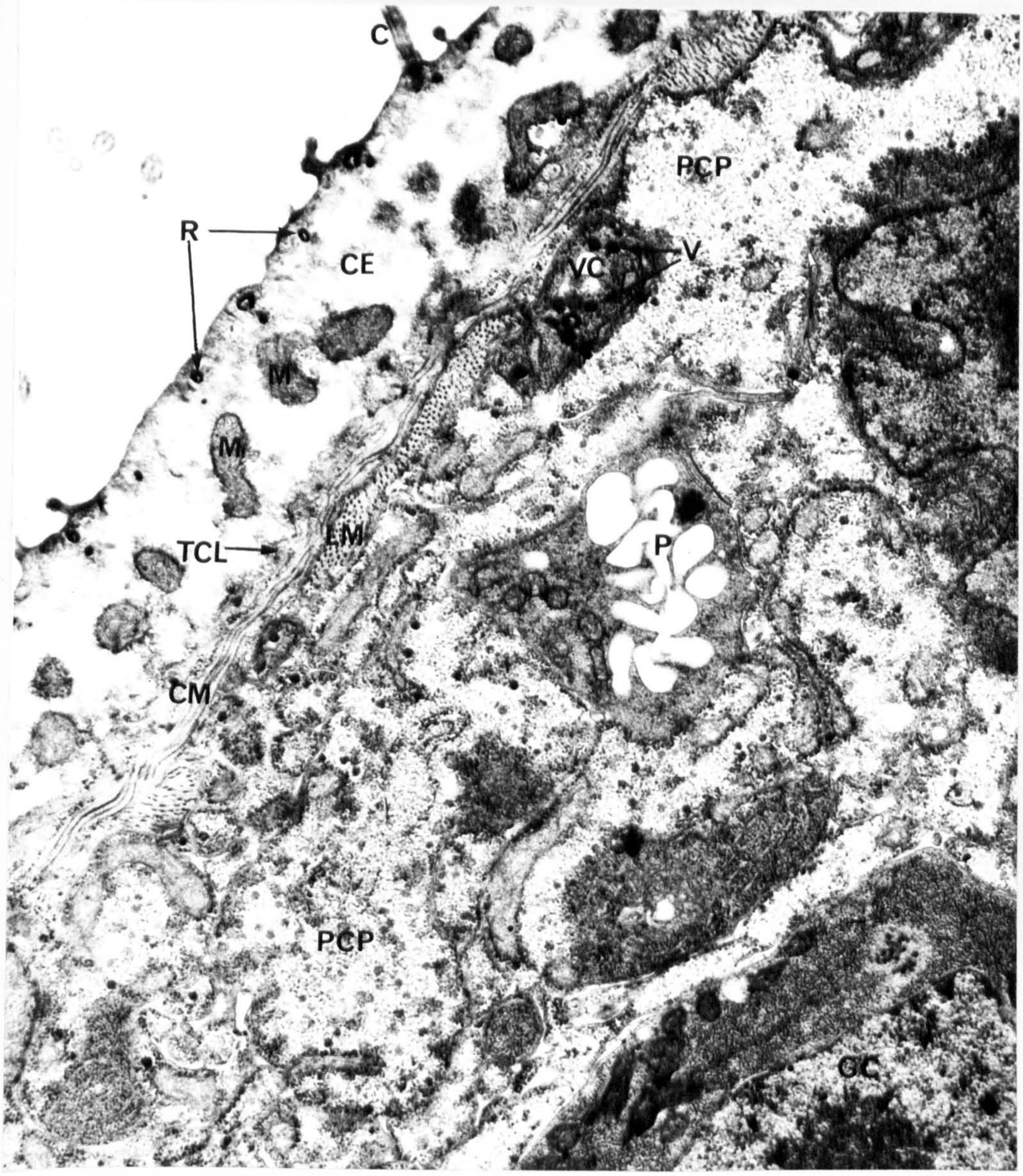
R - rootlets.

TCL - thin cytoplasmic layer.

V - dense vesicles.

VC - vesiculated cell.

(Magnification, x 15,000).



1µm

PLATE 12

Plate 13 Transverse section of a miracidium showing the structure of the outer body wall in detail.

BB - basal body.

C - cilium.

CE - part of an epidermal cell.

CM - circular muscle.

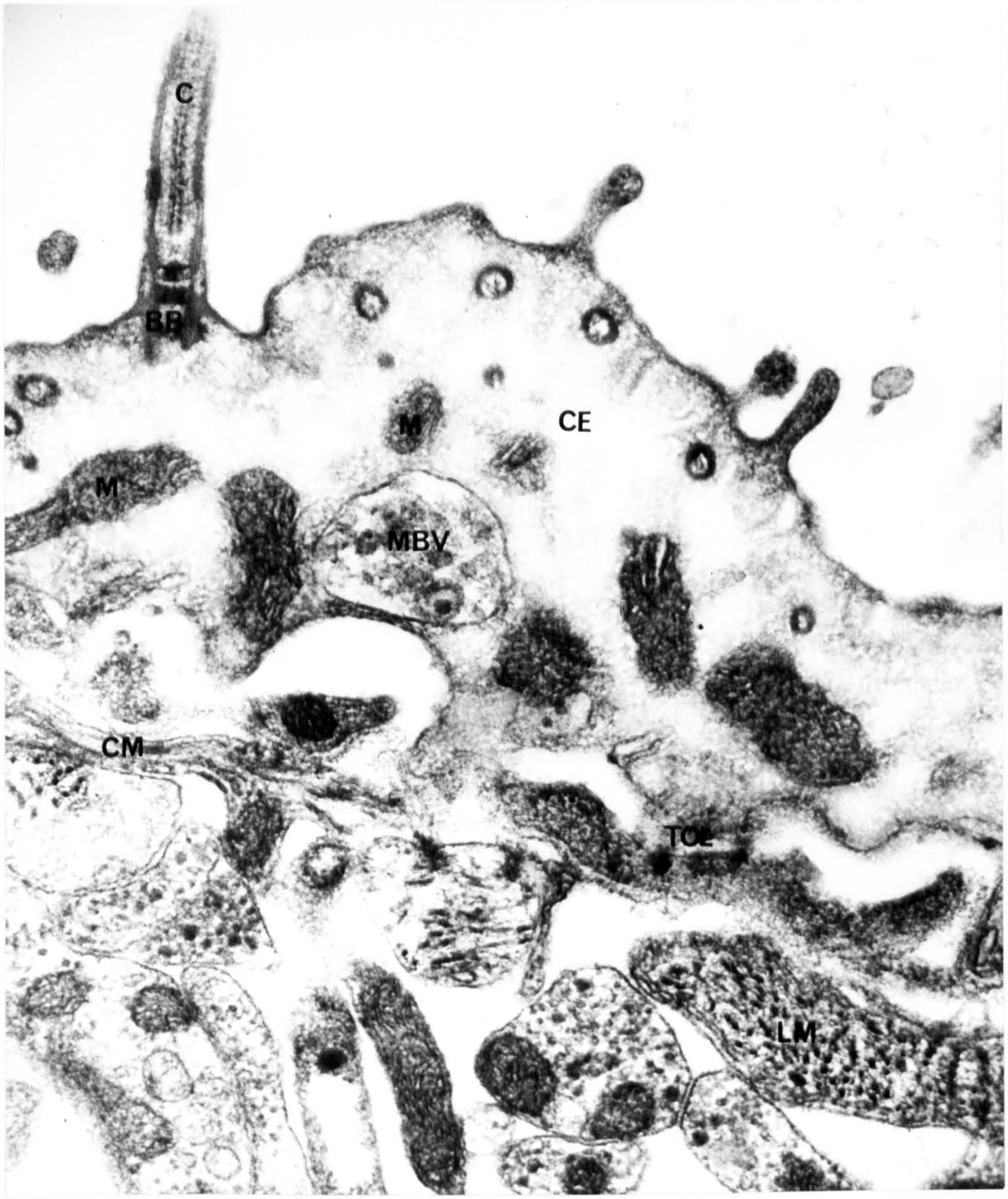
LM - longitudinal muscle.

M - mitochondrion.

MBV - membrane - bound vesicle.

TCL - thin cytoplasmic layer.

(Magnification, x 39,000)



1µm

PLATE 13

Plate 14. Transverse section of a sporocyst dissected from Lymnaea truncatula 9 days after penetration, showing the general structure of the body wall:

BW - body wall with corrugations.

BWR - body wall of a developing redia.

CB - cell boundaries.

CM - circular muscle*

LM - longitudinal muscle*

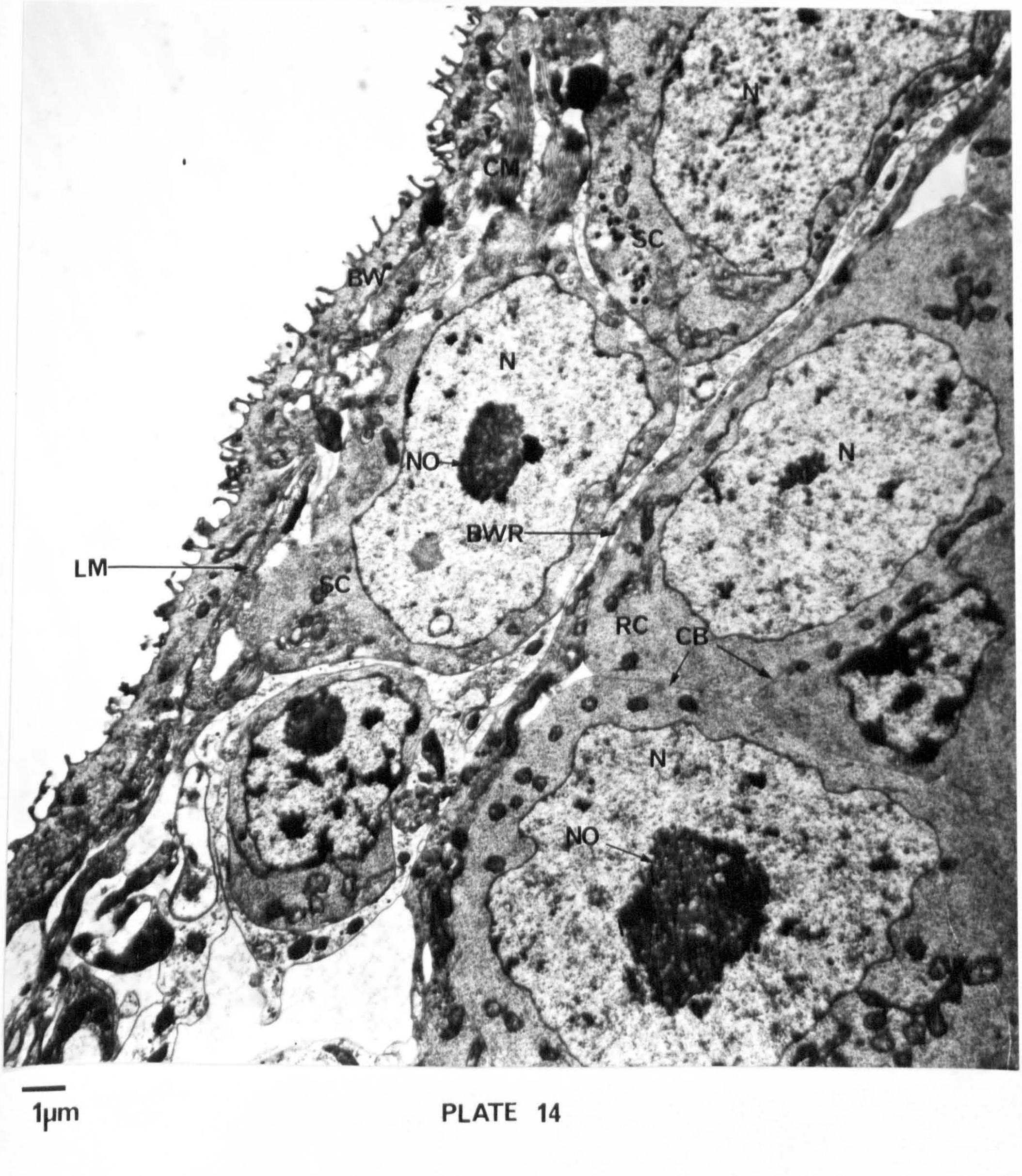
N - nuclei.

NO - nucleoli.

RC - redial cell.

SC - sporocyst cells.

(Magnification, x 6,000) * The arrangement of the musculature is not clear.



1µm

PLATE 14

Plate 15. Transverse section of a sporocyst dissected from Lymnaea truncatula 9 days after penetration, showing the structure of the outer body wall in detail:

BM - basement membrane of body wall.

BW - body wall with corrugations.

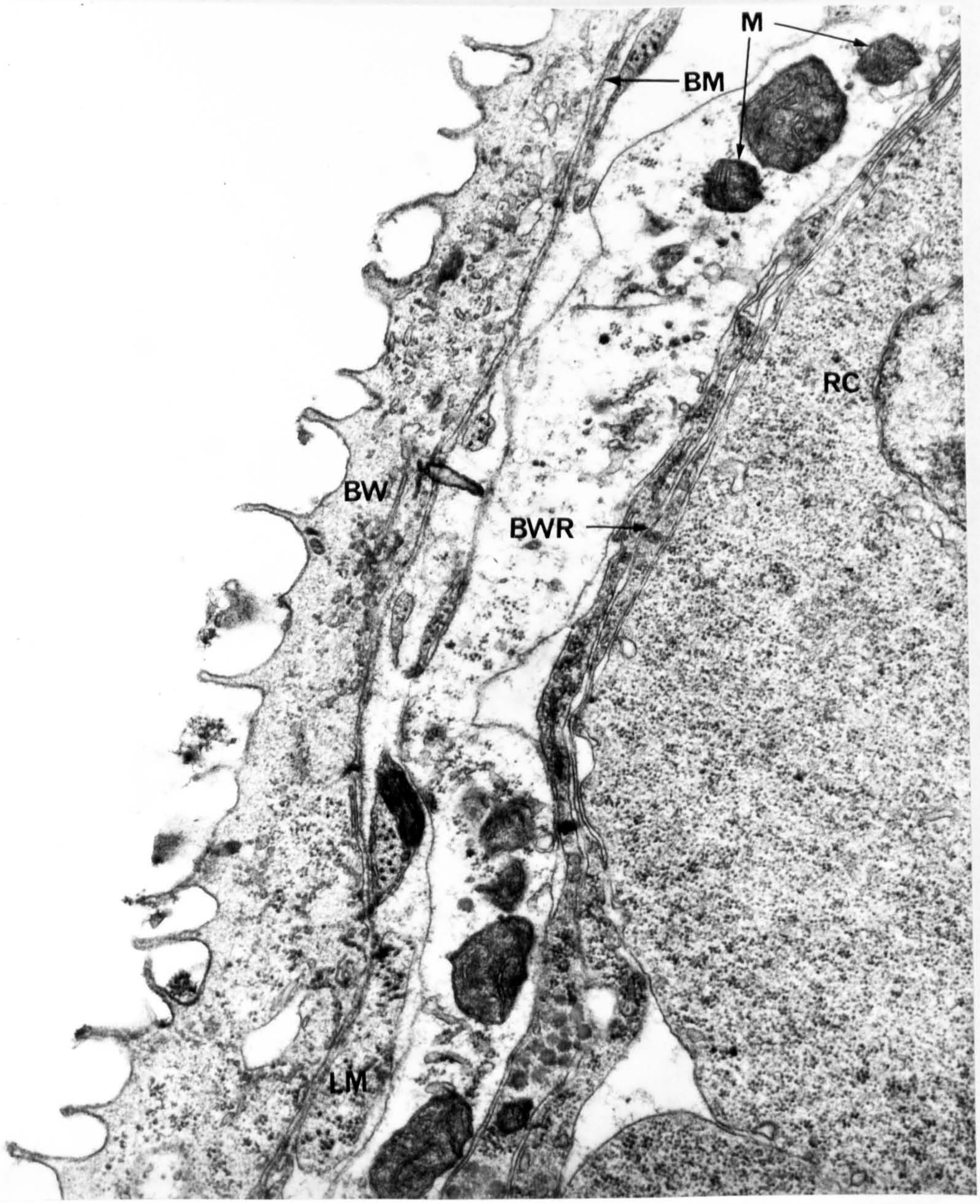
BWR - body wall of a developing redia.

LM - longitudinal muscle.

M - mitochondria.

RC - redial cell.

(Magnification, x 20,000)



1µm

PLATE 15

Plate 16. Transverse section of a sporocyst dissected from Lymnaea truncatula 9 days after penetration showing a break in the basement membrane of the body wall:

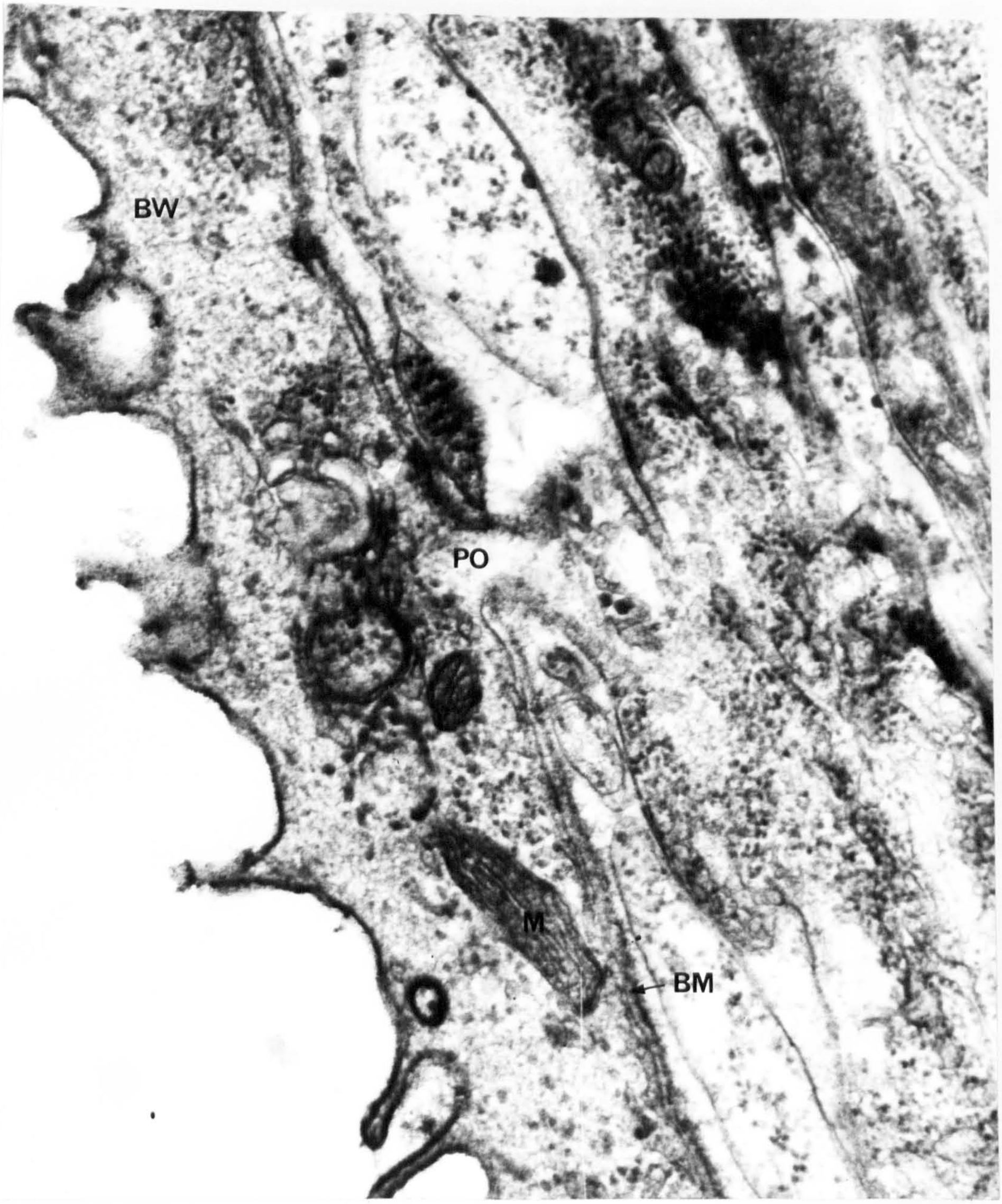
BM - basement membrane of body wall.

BW - body wall.

M - mitochondrion.

PO - break in the basement membrane: a pore.

(Magnification, x 44,000)



1µm

PLATE 16

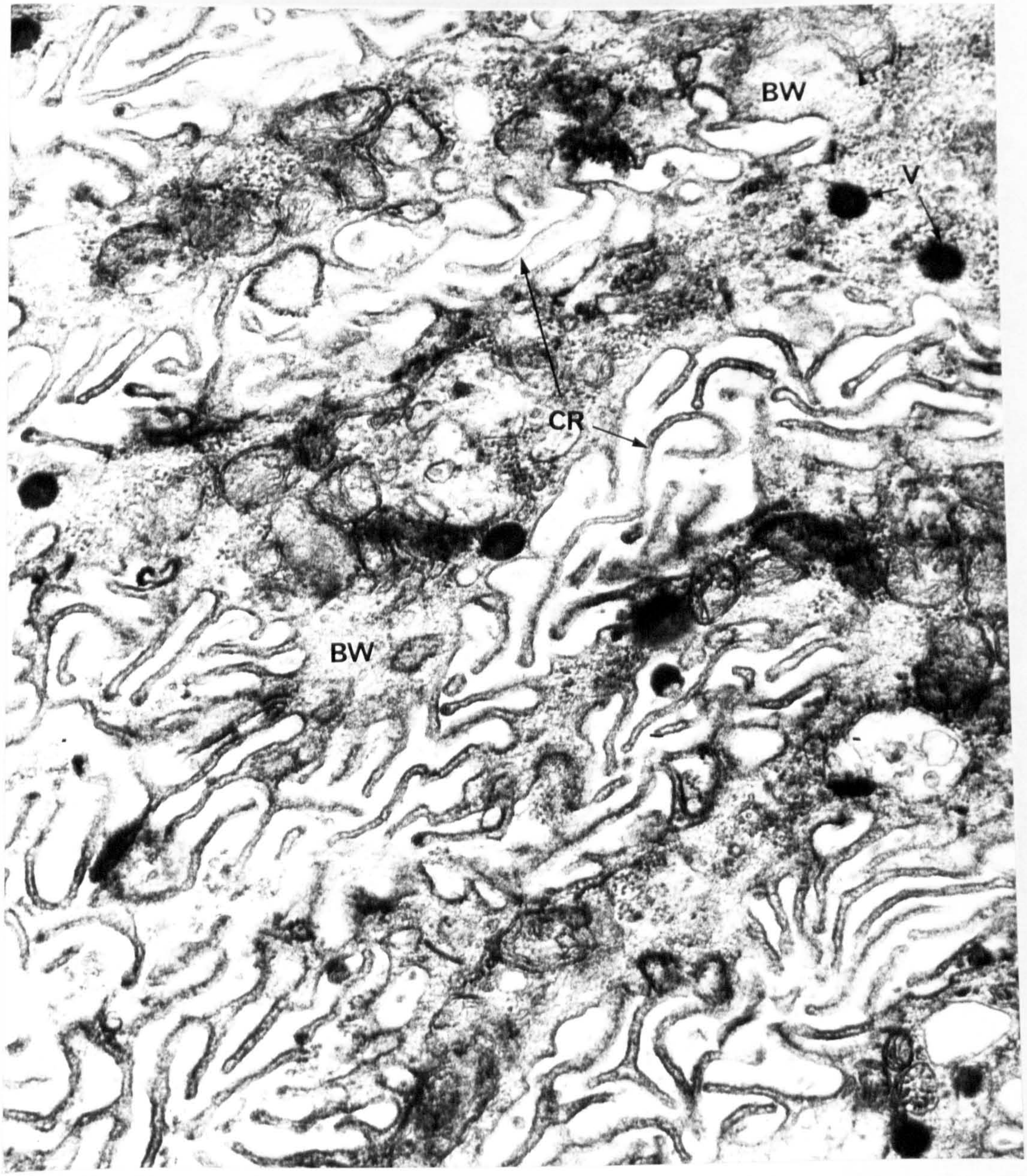
Plate 17. Tangential section across the body surface of a sporocyst dissected from Lymnaea truncatula 9 days after penetration, showing the corrugations of the body surface:

BW - body wall cytoplasm.

CR - corrugations.

V - dense vesicles in or just below the body wall.

(Magnification, x 30,000)



1µm

PLATE 17

Plate 18. ~~Transverse~~ Longitudinal section of a sporocyst 24 hours after hatching and metamorphosis "in vitro", showing the structure of the new body wall in the middle of the body:

BM - basement membrane of new body wall.

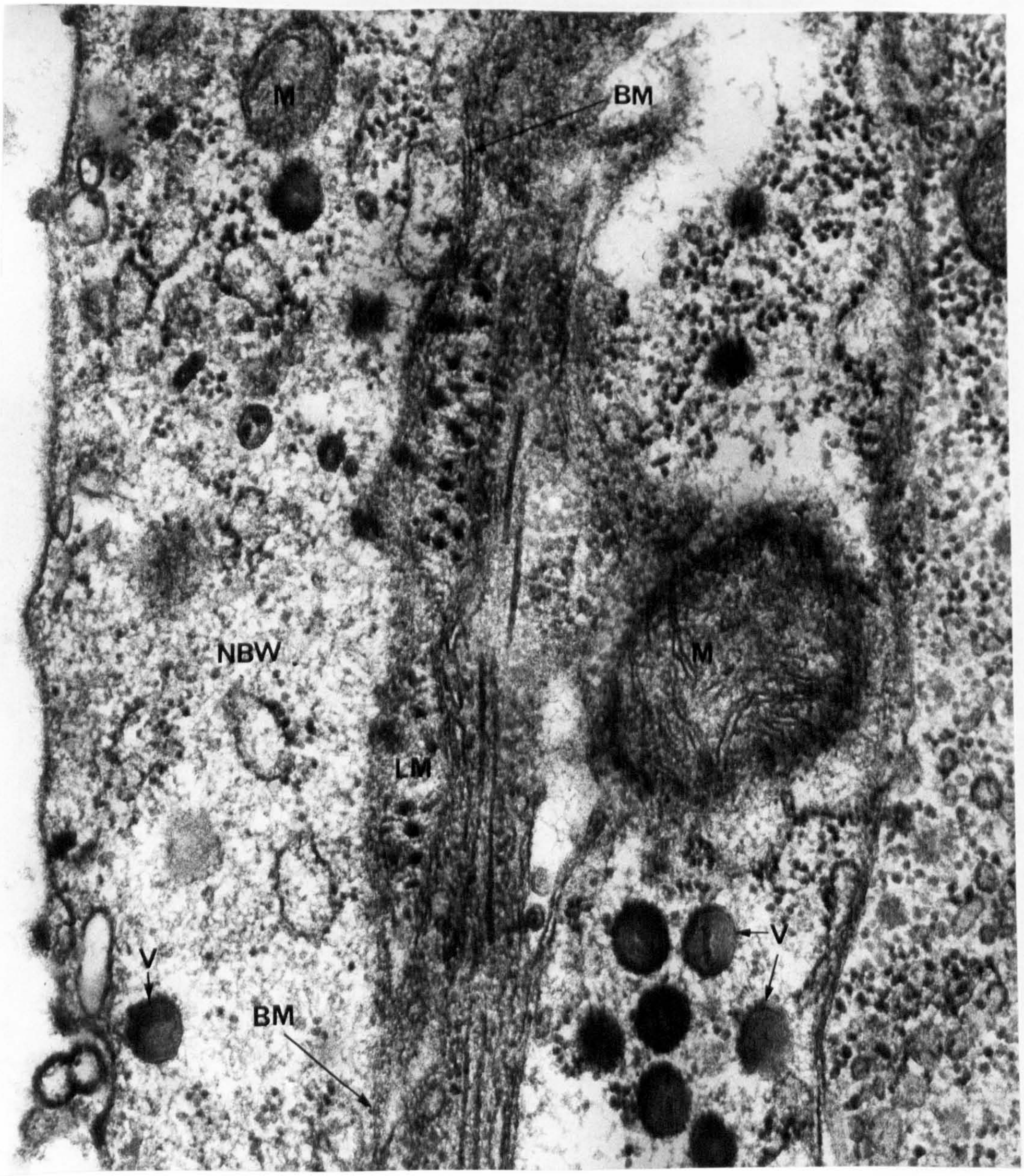
LM - longitudinal muscle.

M - mitochondria.

NBW - new body wall.

V - dense vesicles.

(Magnification, x 54,000)



1μm

PLATE 18

Plate 19. Transverse section of a sporocyst 24 hours after hatching and metamorphosis "in vitro", showing the structure of the new body wall in the middle of the body:

BM - basement membrane of new body wall.

BWC - body wall corrugations.

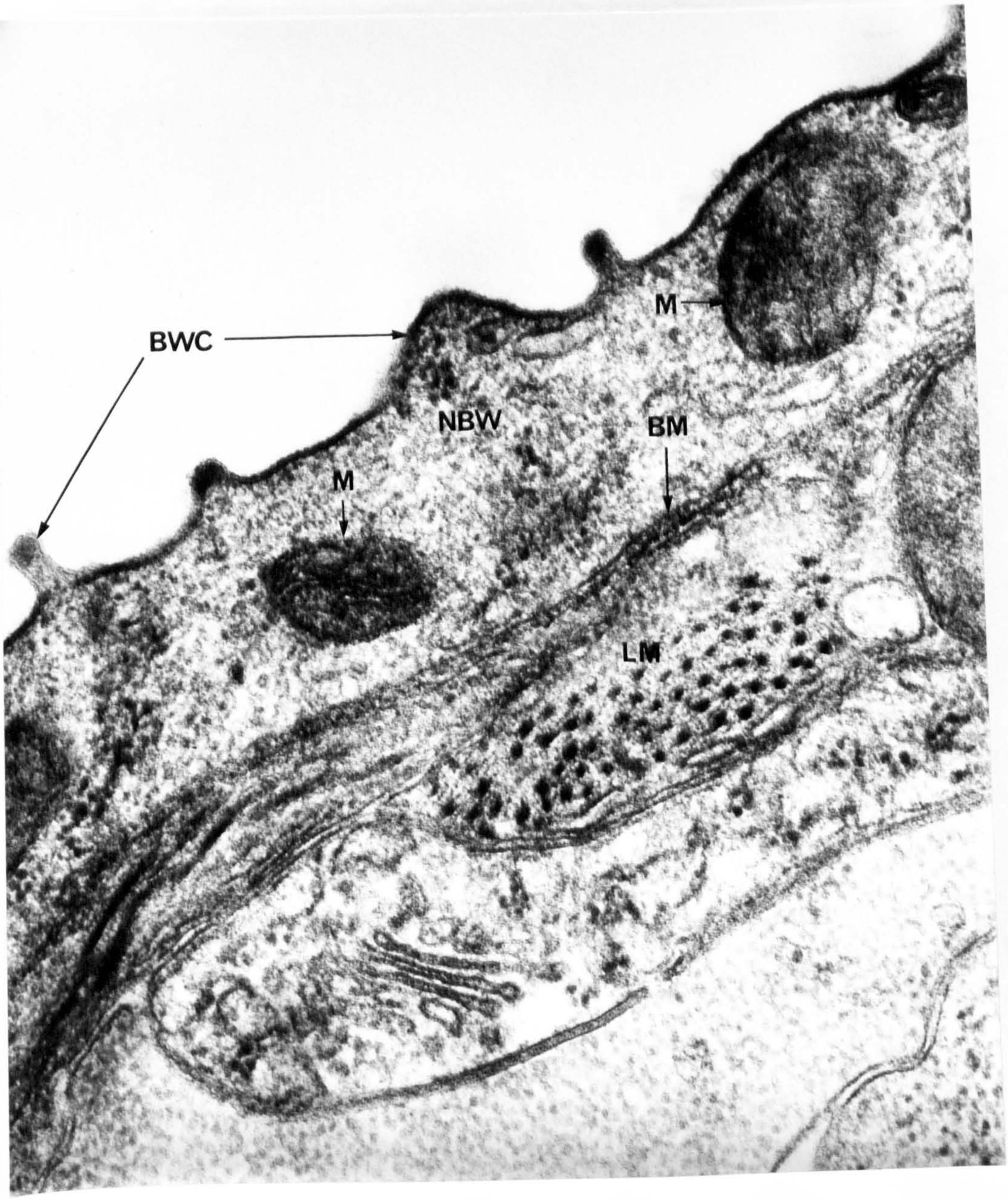
CM - circular muscle.

LM - longitudinal muscle.

M - mitochondria.

NBW - new body wall.

(Magnification, x 59,400)



1μm

PLATE 19

Plate 20. Transverse section of a sporocyst 24 hours after hatching and metamorphosis 'in vitro', showing the new body wall greatly proliferated towards the posterior end of the body.

BM - basement membrane of new body wall.

CM - circular muscle.

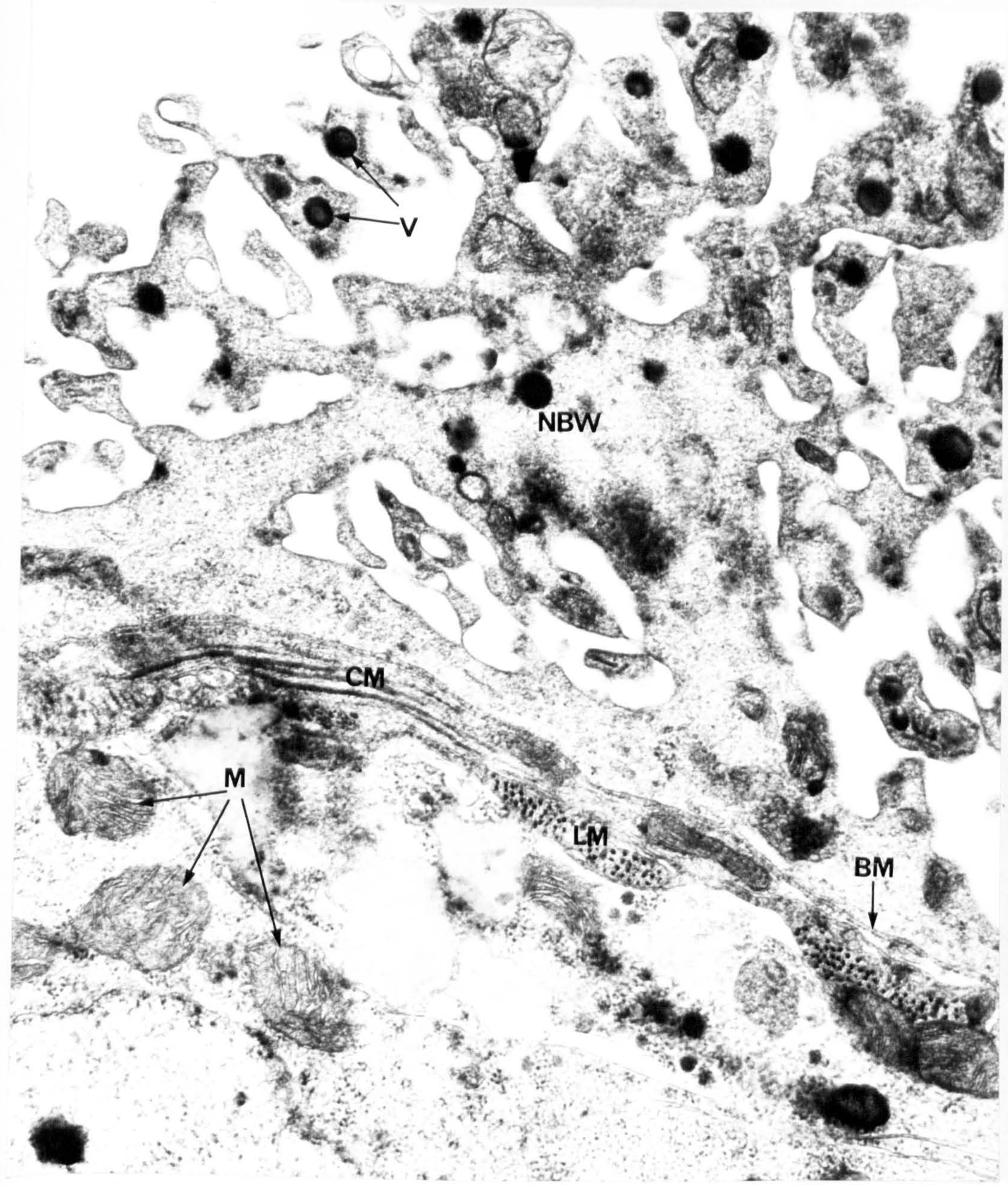
LM - longitudinal muscle.

M - mitochondria

NBW - new body wall.

V - dense vesicles.

(Magnification, x 23,000)



1µm

PLATE 20

Plate 21. Transverse section of a sporocyst 24 hours after hatching and metamorphosis 'in vitro', showing proliferation of the new body wall at the anterior end of the germ cell region:

BM - basement membrane of new body wall.

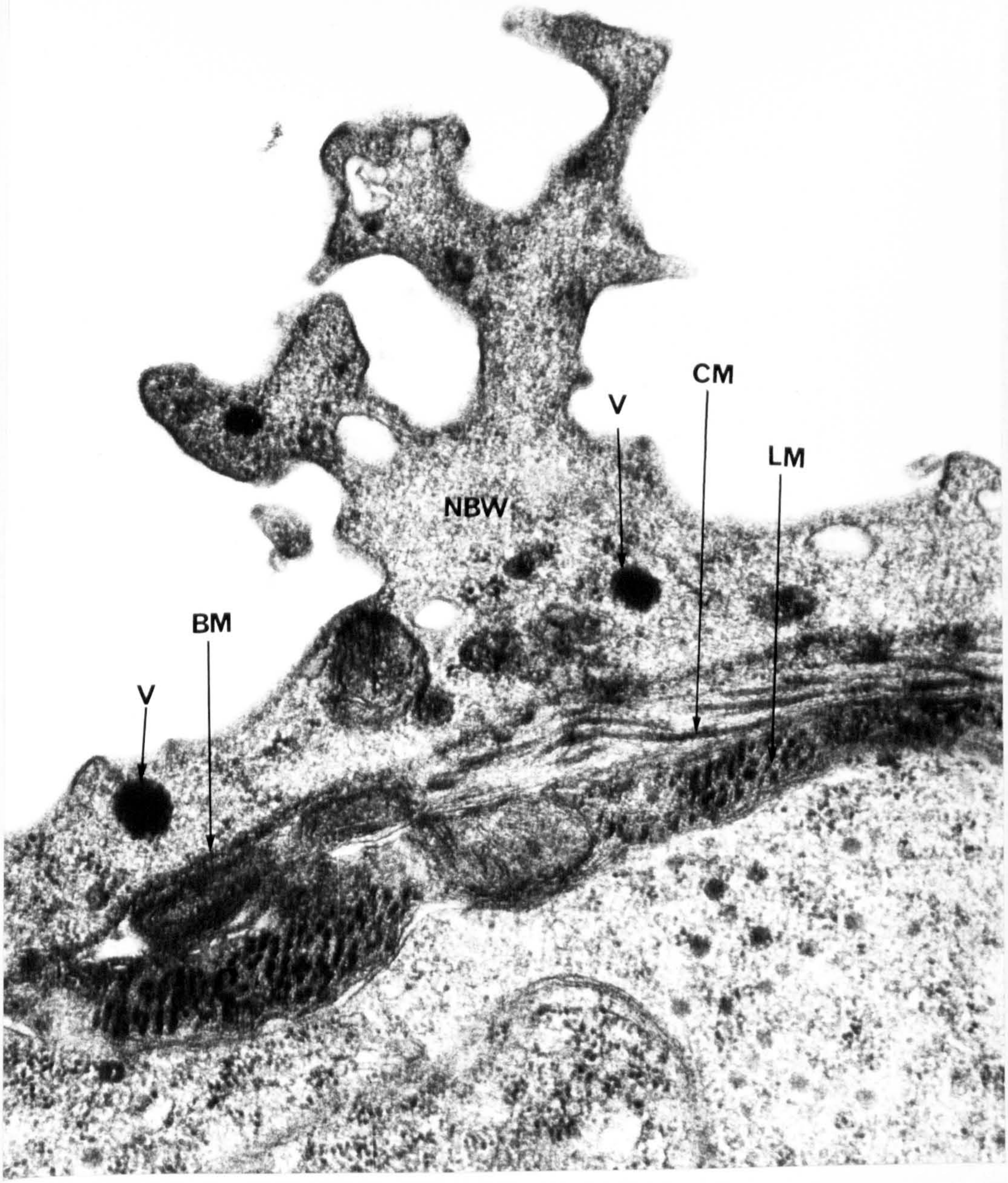
CM - circular muscle

LM - longitudinal muscle.

NBW - new body wall.

V - dense vesicles.

(Magnification, x 34,500)



1μm

PLATE 21

Plate 22. Transverse section of a sporocyst 24 hours after hatching and metamorphosis 'in vitro', showing an area of 'reorganisation' in the anterior portion of the body near to the eyespots:

CD - debris from broken-down cells.

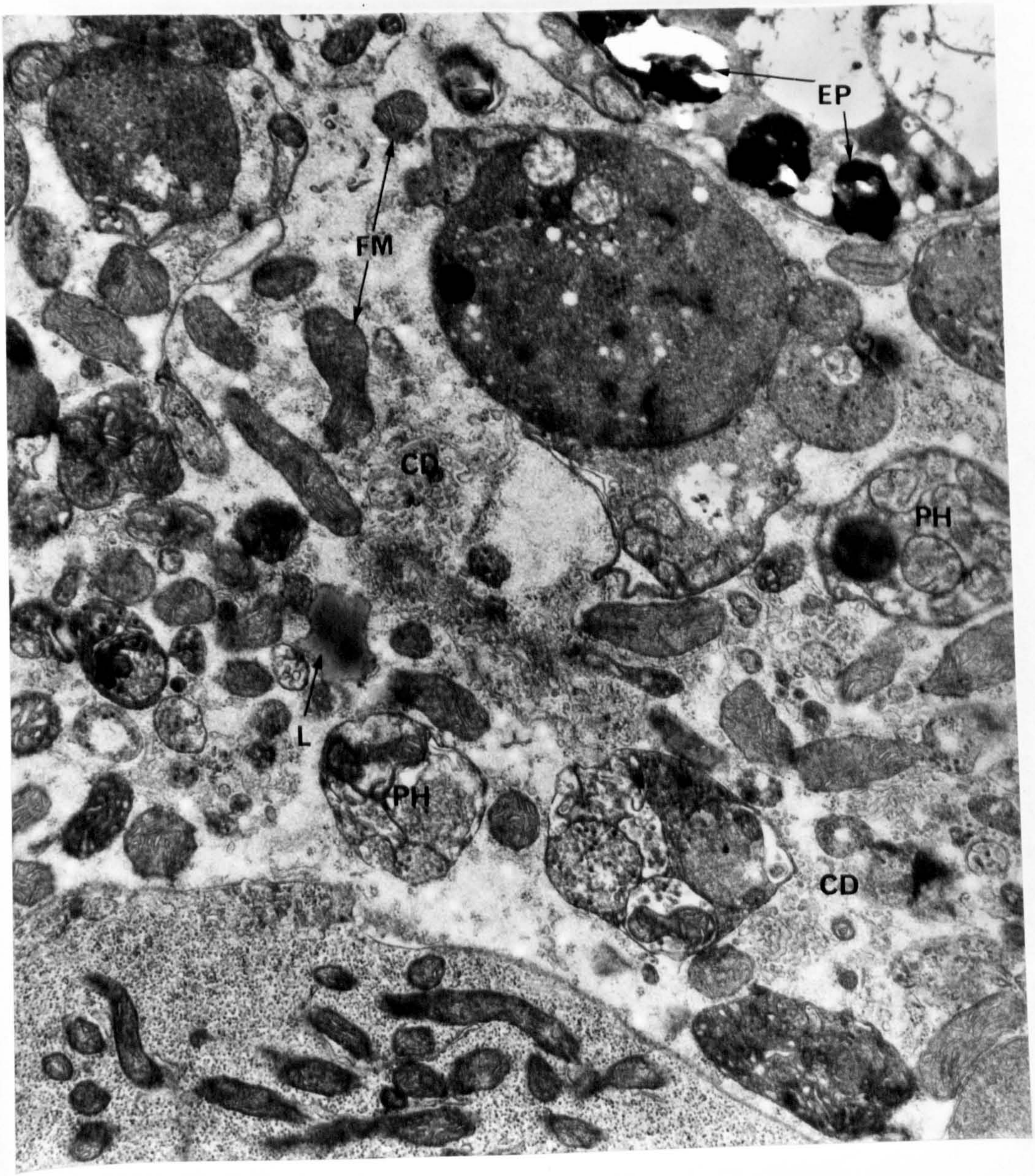
EP - eyespot pigment.

FM - free mitochondria.

L - lipid material.

PH - suggested phagosomes.

(Magnification, x 15,000)



1μm

PLATE 22

Plate 23. Transverse section of a sporocyst 24 hours after hatching and metamorphosis 'in vitro', showing an area of 'reorganisation' near the posterior end of the body:

CD - debris from broken-down cells.

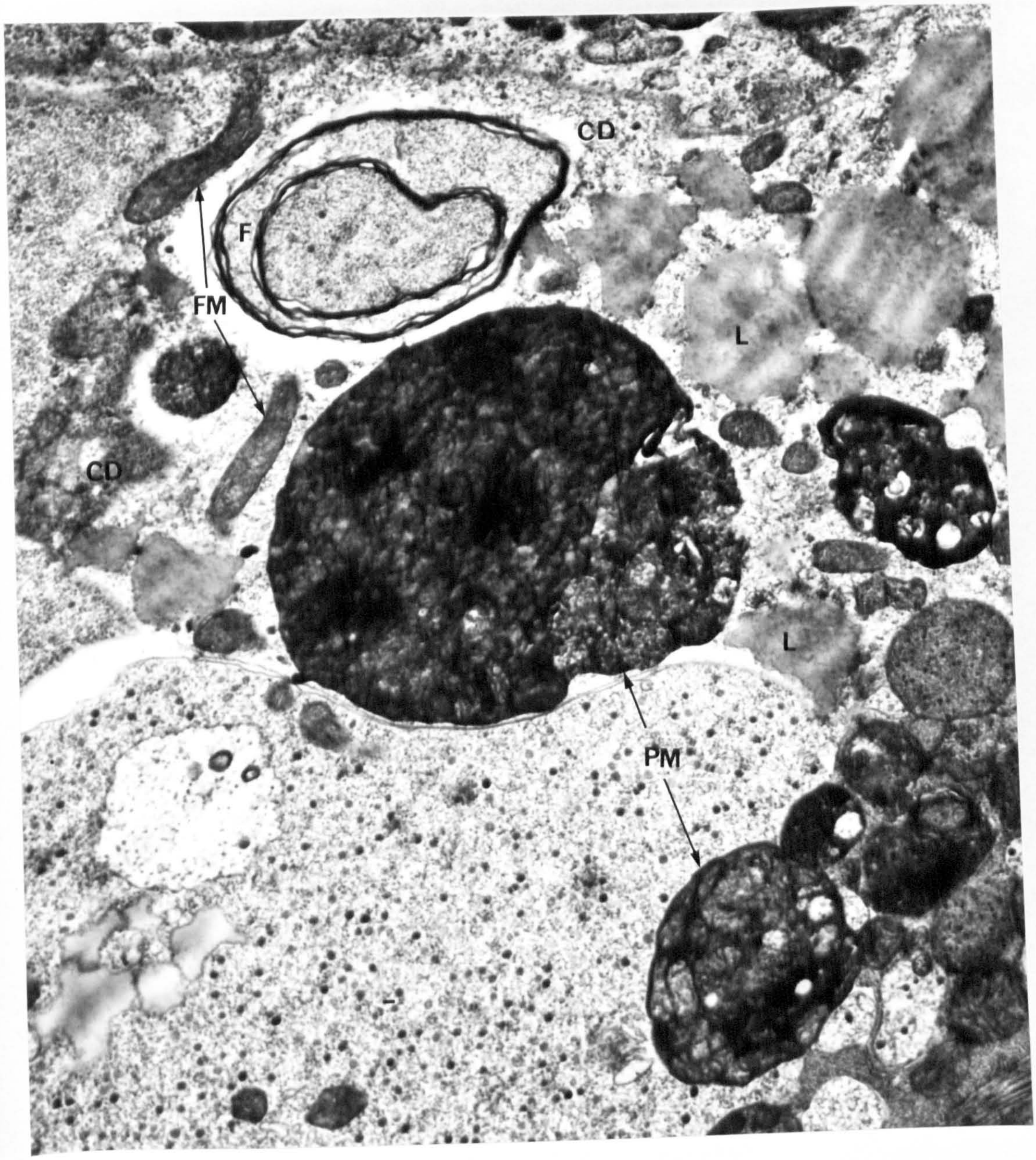
F - a 'fibrous' structure of unknown origin.

FM - free mitochondria.

L - lipid material.

PM - large 'packets' of cellular material (probably mitochondria in phagosomes).

(Magnification, x 11,400)



1µm

PLATE 23

Plate 24. Transverse section of a sporocyst 24 hours after hatching and metamorphosis 'in vitro', showing a clump of germ cells:

CB - cell boundaries

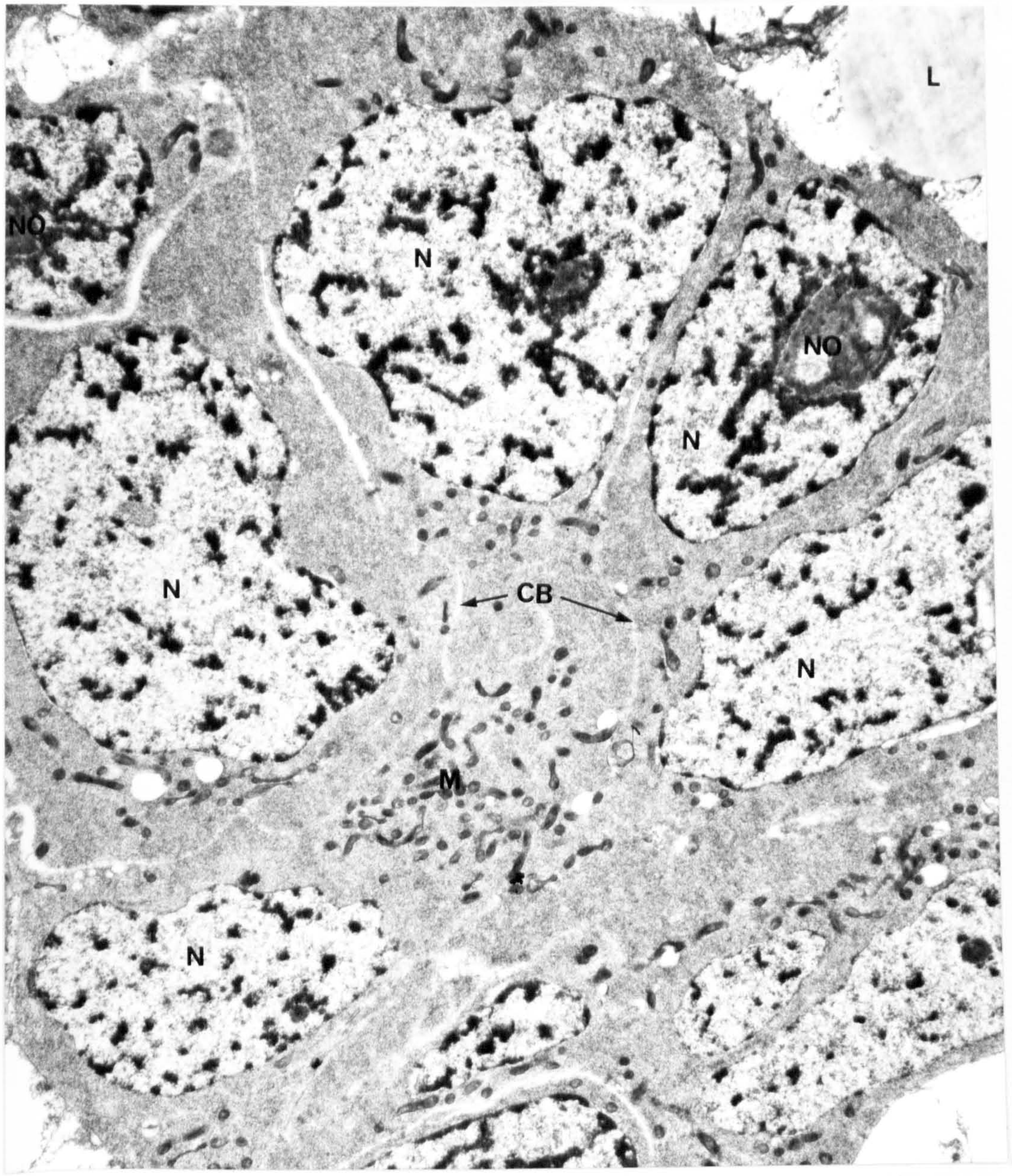
L - lipid material.

M - mitochondria.

N - nuclei

NO - nucleoli

(Magnification, x 20,250)



1μm

PLATE 24

Plate 25. Transverse section of a sporocyst 24 hours after hatching and metamorphosis 'in vitro', showing part of a flame cell:

C - cilia

D - desmosome

IM - inner matrix

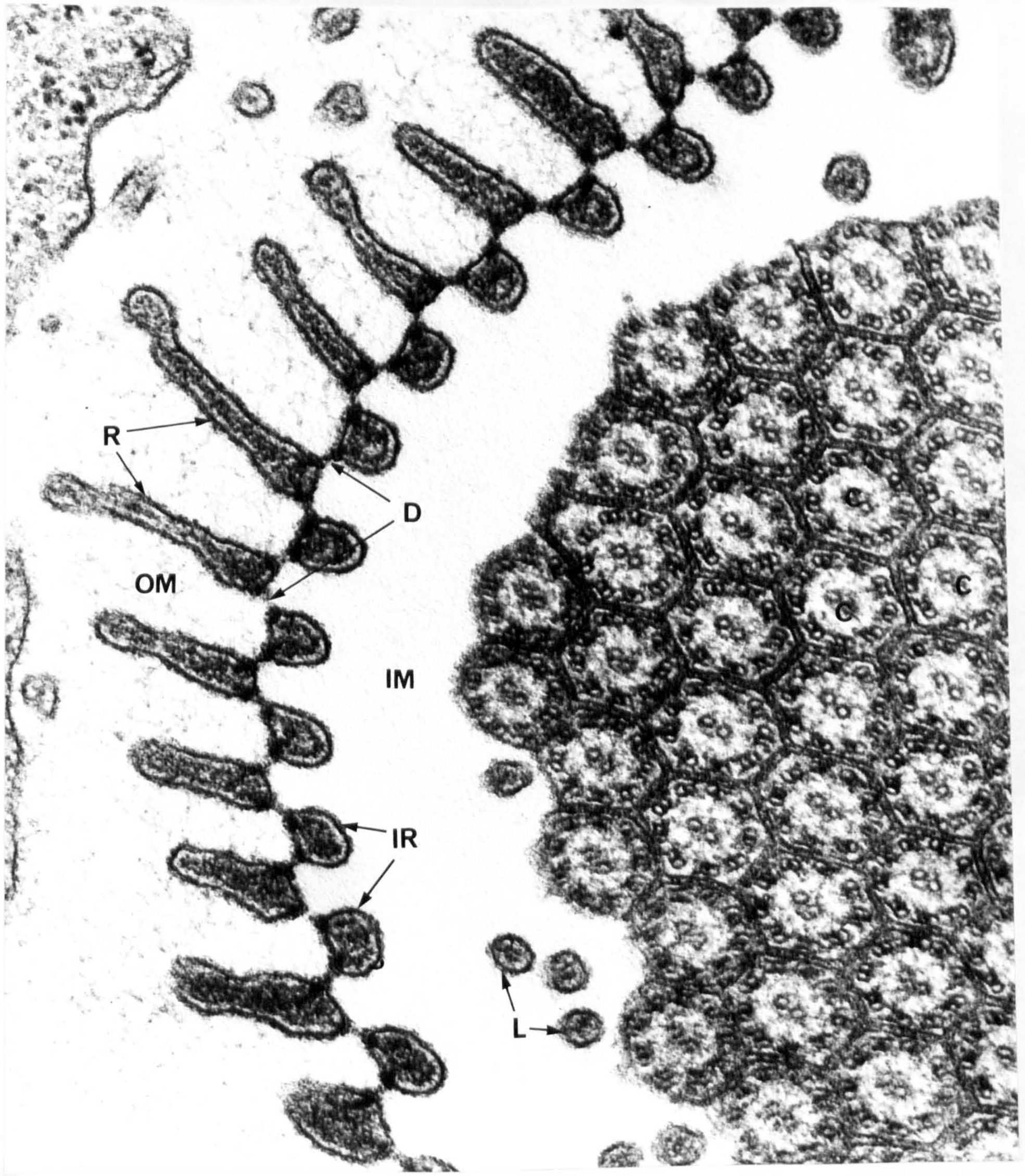
IR - inner ribs

L - leptotriches

OM - outer matrix

R - ribs

(Magnification, x 80,000)



1μm

PLATE 25

Plate 26. Transverse section of a sporocyst 72 hours after hatching and metamorphosis 'in vitro', showing the general structure of the new body wall (which shows no corrugations) and 'reorganisation' in the deeper tissues:

AR - area of 'reorganisation'

CM - circular muscle

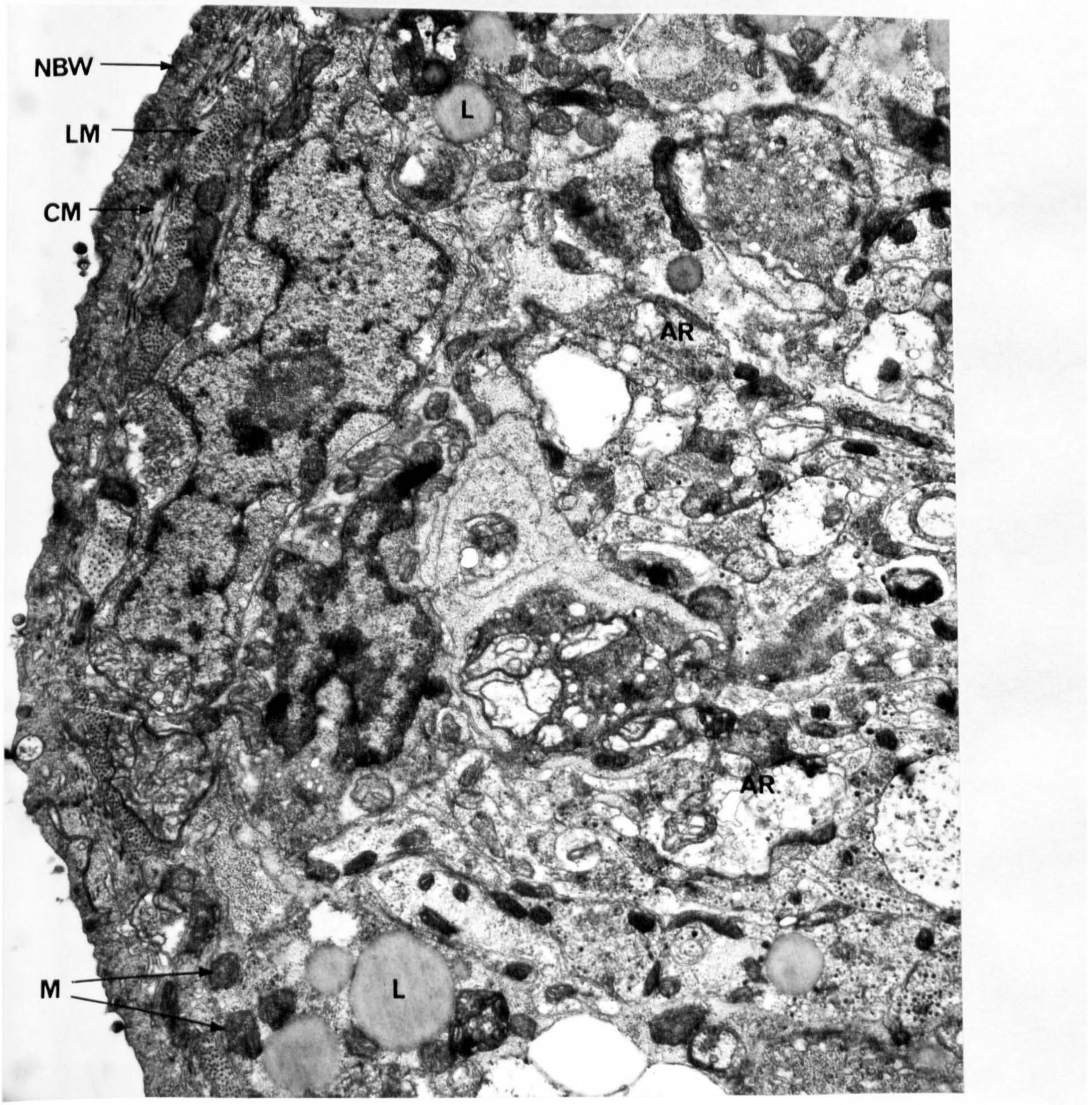
L - lipid material

LM - longitudinal muscle

M - mitochondria

NBW - new body wall

(Magnification, x 11,500)



1 µm

PLATE 26

Plate 27. Transverse section of a sporocyst 72 hours after hatching and metamorphosis 'in vitro', showing the new body wall in detail:

AR - area of 'reorganisation'

BM - basement membrane of new body wall.

CM - circular muscle.

L - lipid material.

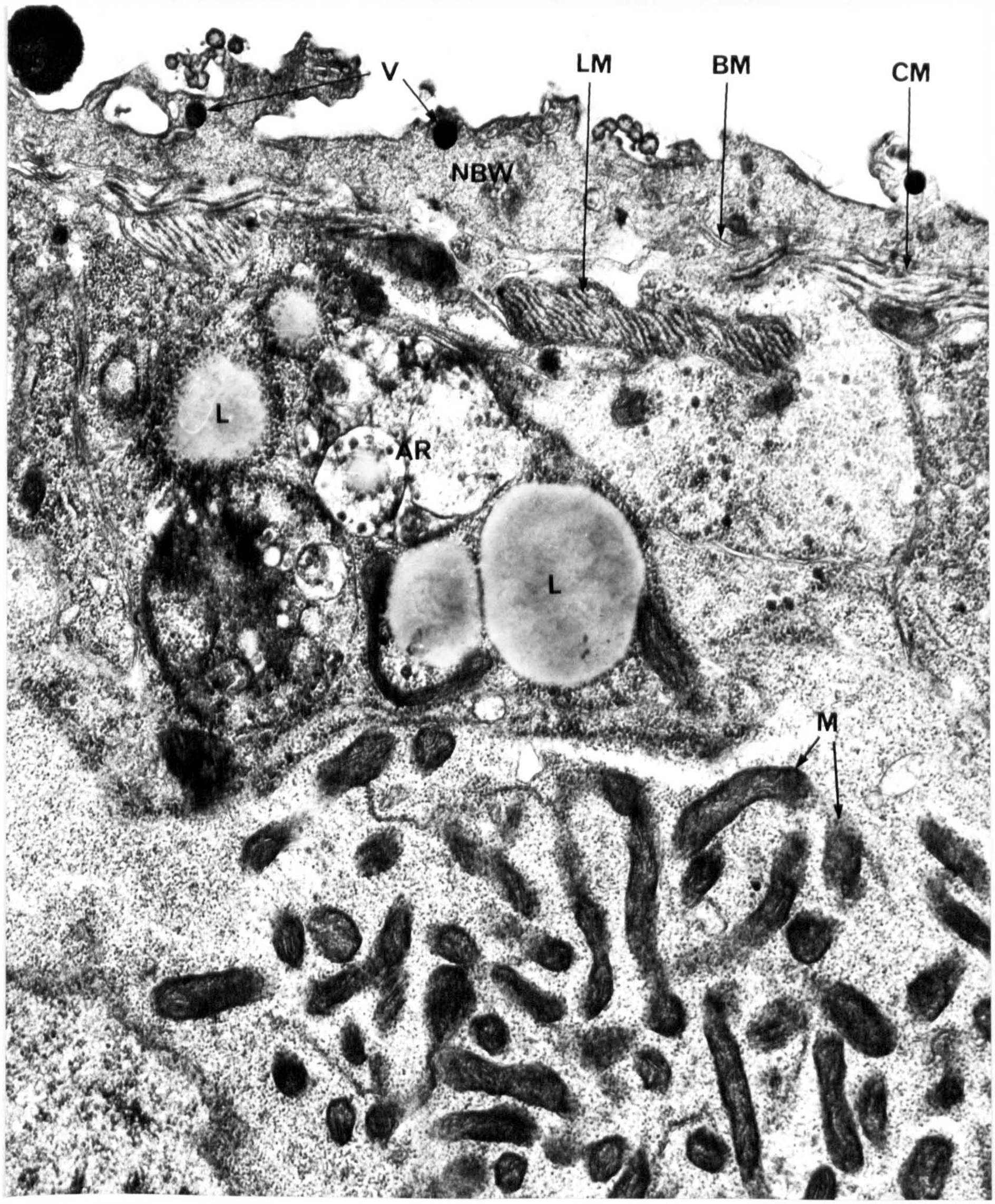
LM - longitudinal muscle.

M - mitochondria

NBW - new body wall with surface irregularities

V - dense vesicle.

(Magnification, x 17,250)



1μm

PLATE 27

Plate 28. Longitudinal ~~Transverse~~ section of a sporocyst, 168 hours after hatching and metamorphosis 'in vitro', showing the structure of the new body wall in detail (the basement membrane is not distinct).

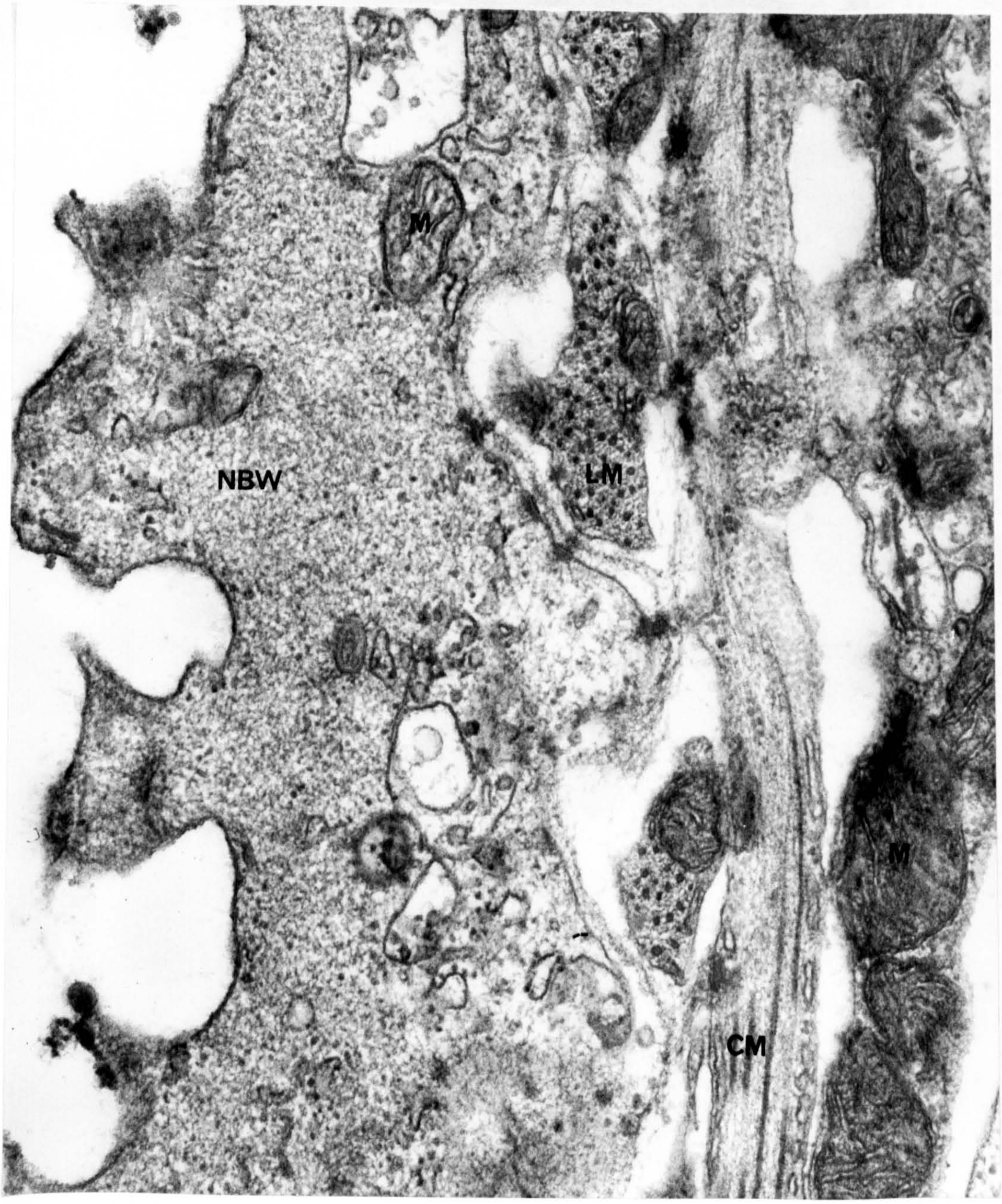
CM - circular muscle

LM - longitudinal muscle

M - mitochondria

NBW - new body wall, with corrugations.

(Magnification, x 41,700)



1µm

PLATE 28

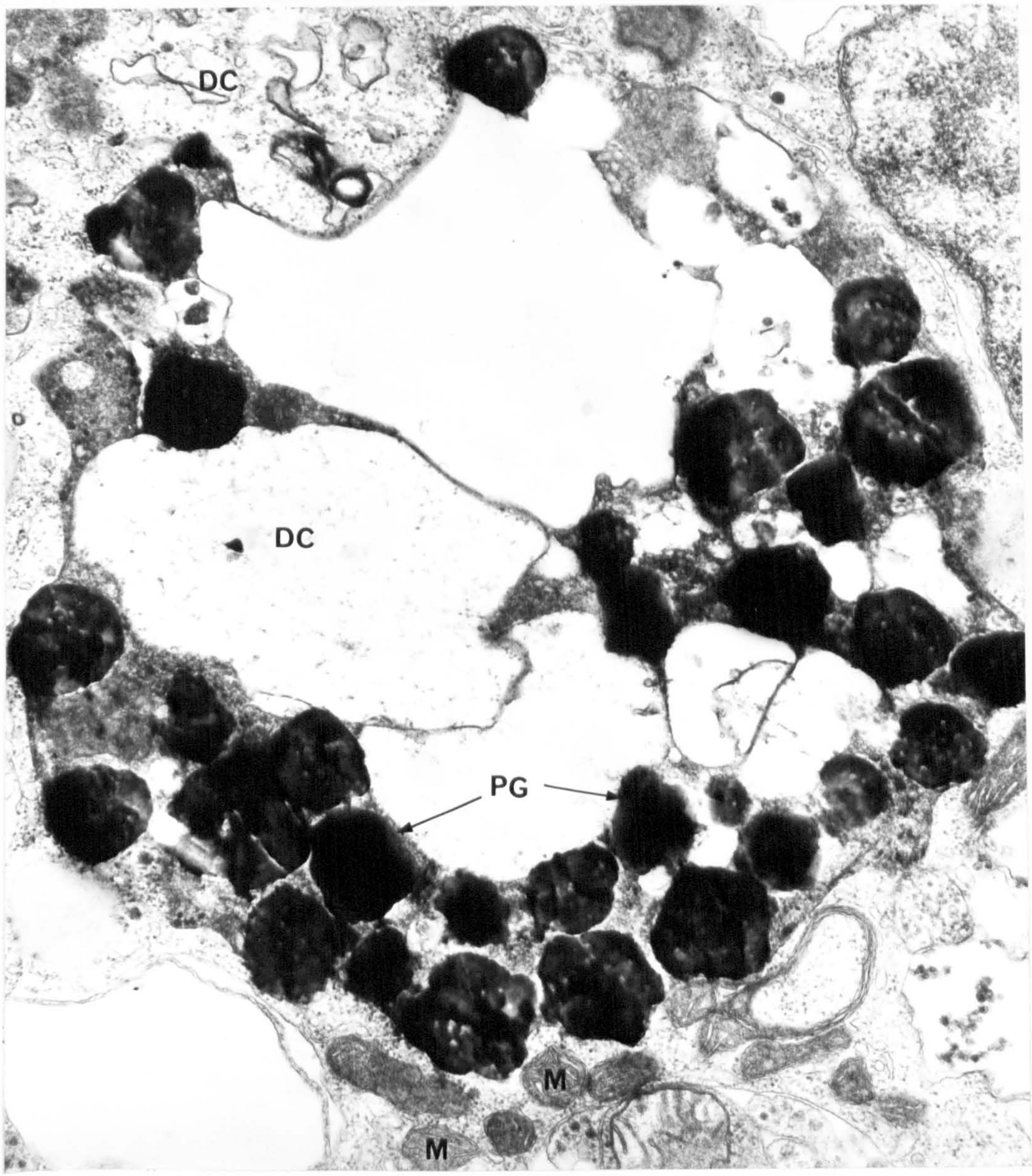
Plate 29. Transverse section of a sporocyst 168 hours after hatching and metamorphosis 'in vitro', showing remains of the eyespot.

DC - disrupted cells

M - mitochondria

PG - pigment granules.

(Magnification, x 19,000)



1μm

PLATE 29

Plate 30. A comparison of the body walls of a) miracidium; b) 'in vitro' cultured sporocyst 24 hours after hatching and c) redia. (all are from transverse sections; the electronmicrograph of the redia was donated by Sommerville, 1966)

a) Miracidium

BB - basal body

C - cilium

CE - parts of epidermal cells

CM - circular muscle

D - desmosome

IR - intercellular ridge

LM - longitudinal muscle

M - mitochondria

R - rootlets

TCL - thin cytoplasmic layer

(Magnification, x 24,450)

b) 'In vitro' sporocyst.

BM - basement membrane of new body wall

CM - circular muscle, which here appears displaced - probably an artefact from sectioning.

LM - longitudinal muscle.

M - mitochondria

NBW - new body wall.

V - dense vesicles.

(Magnification, x 20,000)

c) Redia

BM - basement membrane of body wall

BW - body wall (non-nucleate)

CM - circular muscle

CYT - cytoplasm of muscle cells

LM - longitudinal muscle

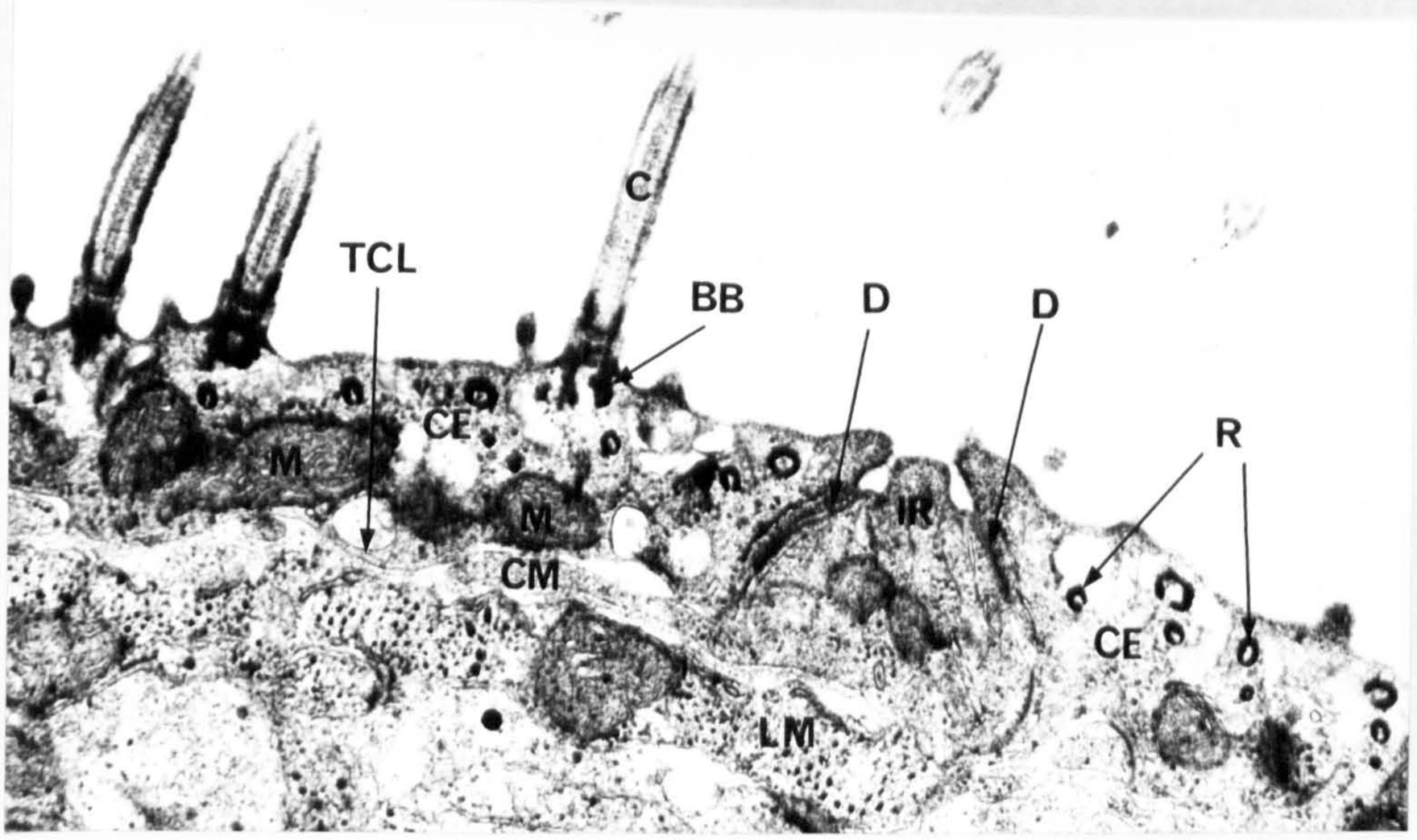
MU - mucus

MV - microvilli

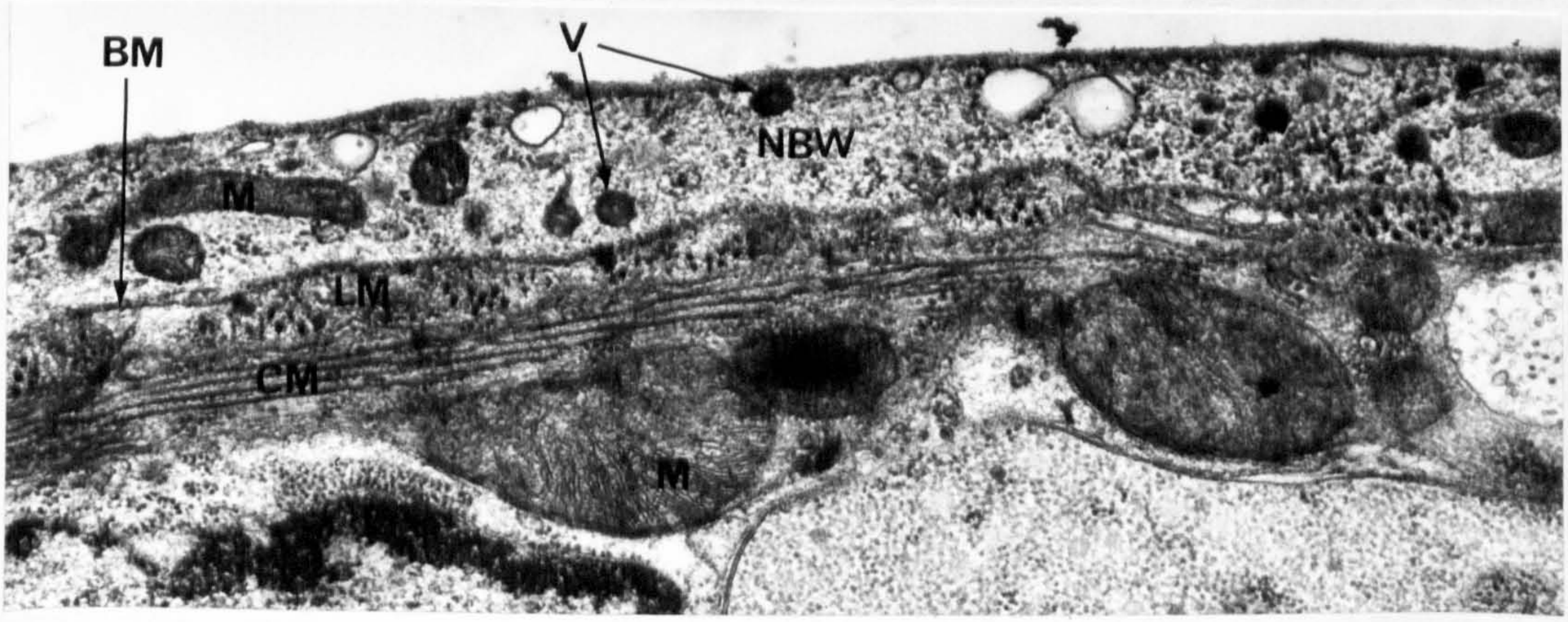
(Magnification, x 11,450)

-

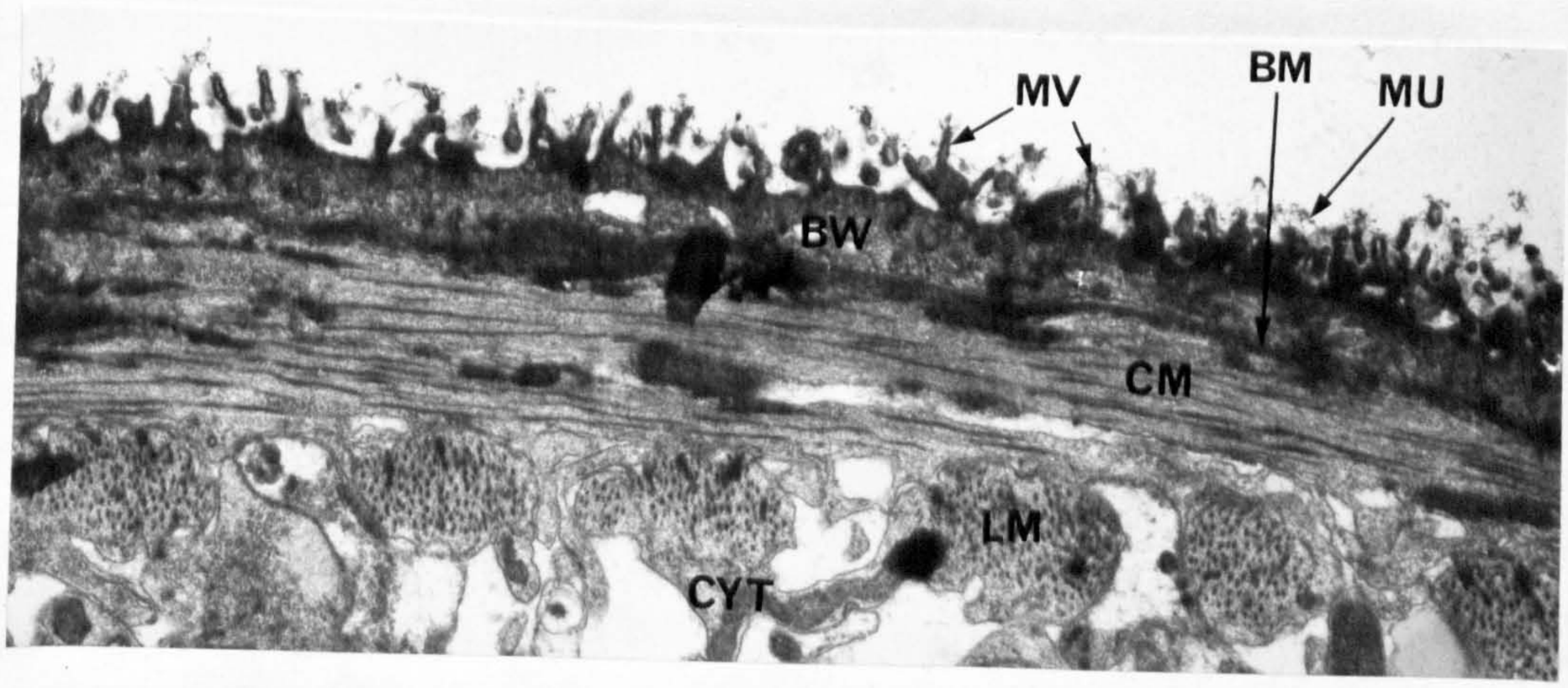
a)



b)



c)



a) ——————
b) —————— 1μm
c) ——

PLATE 30

PART VI The 'in vitro' culture of rediae.

Introduction

As with sporocysts, there has been very little work done on the "in vitro" culture of rediae. Friedl (1961a, 1961b) has described attempts to culture the rediae of Fascioloides magna in a variety of non-defined media and Ingersoll (1956) has described "in vitro" survival of the rediae of Cyclocoelum microstomum which occur ready-formed in the miracidia. Nothing more than survival of rediae "in vitro" has been achieved as yet.

With the limited time available for the present study, it was decided to attempt to culture rediae, dissected from infected snails, in sterile BCM. Despite the inadequacy of BCM in supporting growth and development of sporocysts and the information given by Cheng (1963) concerning lipids, it was felt that rediae could possibly grow well in a simple medium. Rediae are well-acclimatised to an environment close to the pH and tonicity of haemolymph and they have no metamorphoses to perform: remembering that metamorphosis from miracidium to sporocyst is associated with a drastic change in environment (from fresh water to haemolymph). Rediae could merely require the right amino acids and a suitable carbohydrate source from which to synthesise cercarial tissue. The adverse possibilities are that rediae may require both lipids and proteins to complete

this synthesis; the latter both as a physical constituent of the environment and as solid food. It may be that rediae cannot survive without using their gut. With this in mind, brief attempts are described here to supplement BCM with gelatin, collagen, and whole haemolymph from Lymnaea stagnalis. The use of all three supplements really "broke the rules" of the present study, producing non-defined media. The use of haemolymph was, however, merely undertaken as an empirical trial which, if successful, could have been followed by separation and purification of haemolymph fractions.

The original idea that "in vitro" culture of larval trematodes should be easy comes from Smyth (1962) who states that sporocysts and rediae occur in sterile sites in the molluscan hosts and should readily lend themselves to axenic culture attempts. Some of the work described in this part, suggests that the digestive gland may not be a sterile site.

Culture experiments were planned with reference to the work of Sommerville (personal communication, 1966) on the growth and development of the rediae of Fasciola hepatica 'in vivo'. This would have allowed a very interesting comparison of growth 'in vivo' and "in vitro", had the latter been more successfully achieved. A summary of Sommerville's findings are given in Appendix II.

Techniques are described here for dissecting so-called sterile

rediae from infected snails, together with an account of criteria developed for assessing possible growth and development "in vitro". An account follows of the two main culture experiments, and of the work with supplemented BCM.

Methods

1.) Techniques for obtaining sterile rediae.

100 snails of total shell length 5 to 6 mm were exposed to 5 miracidia each for 3 hours. During this period, the snails were kept in individual solid watch-glasses, covered with glass plates, containing aerated tap water. Infected snails were maintained on flourishing algal cultures at a known constant temperature (usually 20°C) in a cooled incubator. Sommerville (personal communication, 1966) has shown that the greatest period of redial growth - i.e. the greatest demonstrable increase in dry weight - occurs during the last few days before cercarial rediae begins. This period is normally from 30 to 35 days after infection (see Appendix II). Thus infected snails were kept for up to 30 days before dissection with a view to providing large rediae for culture in an active phase of growth.

Before dissection the snails were cleansed from mud and algal debris etc., and were grouped in covered crystallising dishes on moist filter-paper. The sterile glove-box was prepared as described

for sporocyst cultures (Part V, Methods), and the crystallising dishes and all apparatus required for dissection and setting-up the cultures were introduced through a port. During the final phase of sterilisation with U.V. light, the crystallising dishes were covered with sterile Aluminium foil to shield the snails.

Snails were taken and dissected one at a time. The snail was first rinsed in 70% alcohol for 5 seconds and then blotted dry with sterile tissue paper. This was thought to provide adequate surface sterilisation of the shell. The head-foot, however, understandably contracted and probably retained some non-sterile mucus throughout the dissection. The snail was immersed in sterile BCM in a 2 inch glass petri dish inner and the shell crushed gently with a sterile glass rod (as described in Part II for dry weight determinations). Shell fragments were removed, and the rediae teased from the digestive gland using fine sterile needles. Rupture of the digestive gland was kept to a minimum, and a fresh petri dish was used for each dissection.

Freed rediae were transferred by pipette to sterile BCM in a 50 ml conical flask, and were shaken gently in 3 changes of sterile BCM before transfer to the culture vessels. Instruments were immersed in 70% alcohol between dissections, and apparatus was initially sterilised in metal cans at 160°C for 3 hours.

As a further precaution, the metal cans, and the crystallising dishes containing the snails, were swabbed with 70% alcohol before introduction to the glove-box. The procedure was thus very time-consuming. In addition to the time taken in ensuring sterile conditions, each dissection took approximately 5 minutes. It was found that about 9 hours working inside the sterile glove-box was normally required to provide 250 selected rediae for a culture experiment: a most exhausting procedure.

2) Criteria for assessing growth and development of rediae.

The problems that arose in finding criteria to assess the growth of sporocysts "in vitro" (see Part V, Methods) do not all apply to rediae, the latter being of much larger size. It was felt that as many criteria as possible should be developed, and that these should include chemical as well as physical measurements. Mere survival "in vitro" could be judged by movement, flame cell activity etc. A scheme was devised to determine the dry weight of groups of rediae and to determine the total nitrogen and total carbohydrate in the dried material. The dry weights could then be compared with data on "in vivo" rediae (Sommerville, personal communication, 1966).

For dry weight determinations, groups of rediae were first measured using a calibrated microscope. As with sporocysts, the plastic nature of rediae and their constant movement made these measurements very difficult, and probably rather inaccurate. Measurements made were, 'length when extended' and 'mean breadth when extended' each calculated to the nearest 0.01 mm. 15 to 20 rediae, grouped on size, were lifted from the culture medium using a cut-down camel hair brush, and were placed on a weighed disc of aluminium foil. The foil discs were cut using a paper-punch. The redial samples on the discs were dried overnight at 105°C and allowed to cool in a dessicator before re-weighing. All weighing was performed on the 1 mg scale on the Cahn gram-electro-balance using another foil piece as a counterweight.

For total nitrogen determinations the method of Jacobs (1960, 1962) was used as in Part III, 11.). The fact that the sample was a dried smear on aluminium foil presented no problems. It was shown by trial determinations that the foil contained no nitrogenous impurities and did not produce high blanks. It did, however, produce a white precipitate of aluminium sulphate during the digestion procedure. Thus the transfer of digests to the volumetric flasks was complicated by centrifugation and washing procedures. After initial dilution and centrifugation of the digest and transfer of the supernatant, the precipitate and ampoule were washed 3 times

with distilled-deionised water and the washings added to the flask. Reagents, apparatus and colour development were identical to those in Part III 11.), but here the colours were diluted 1:1 or 1:3 with 50/50 ethanol/water before reading at 570 mμ on the Unicam 5.P. 500 spectrophotometer.

The determination of total carbohydrate presented rather more difficulties. The basic method chosen was that of Kahan (1953) to determine here essentially glycogen + glucose. The dried redial sample on the foil was homogenised in 0.25 ml 5% aqueous trichloro-acetic acid in a small M.S.E. 'Tefluon' homogeniser tube. Careful control of the speed allowed the foil piece to be ground up without developing excessive heat. The homogenate was transferred to a conical centrifuge tube, and the homogenising apparatus washed out with a further 0.25 ml 5% aqueous trichloroacetic acid and 0.2 ml 0.3N barium hydroxide solution; adding the washings to the centri-fuge tube. The washings were mixed on the 'Whirlimixer' (Fisons) and allowed to stand for 5 minutes before adding 0.2 ml of a 5% zinc sulphate solution, previously matched to the barium hydroxide so that equal volumes were exactly equivalent (Somogyi, 1945). After a final mixing and centrifugation the supernatant provided 0.5 ml aliquots for carbohydrate determinations. The 2 deproteinisation

procedures were possibly not necessary as the heat treatment when drying the rediae would render most of the protein insoluble.

Attempts to follow Kahan's (1953) scheme for colour development met with great difficulties. When attempting to construct a calibration curve it was found that Kahan's step 5. was very difficult to standardise and that the heating on tubes on a boiling water bath was <u>essential</u> for any colour development, with the amounts of carbohydrate present in the redial samples. Wellcome chemical control serum provided a suitable protein-containing glucose standard. On the first trials, blanks and standards all showed turbidity and the optical densities read were meaningless (Table 12a).). After much experimentation with Kahan's method and with modifications used by Marsden (personal communication, 1968), a satisfactory calibration curve was obtained with good linearity (Table 12b.)). The main modification used was the omission of an ethyl acetate phase. The procedure was as follows. Triplicate sets of Wellcome chemical control serum standards were set up containing 36, 72 and 108 µg glucose in 0.15 ml distilled-deionised water, together with triplicate blanks containing water alone. After de-proteinisation as described above, 0.5 ml aliquots of the supernatants were transferred to wide diameter boiling tubes; cooled in ice; and left for at least 10 minutes. For colour development each triplicate set was processed in turn, running a blank tube concurrently. 2.5 ml of 0.2% anthrone (B.D.H.) in M.A.R. sulphuric

acid (B.D.H.) was added to each of the 4 tubes, layering the solution in slowly. The tubes were taken singly from the ice-bath and mixed rapidly for 10 seconds on the 'Whirlimixer' (Fisons). The tubes were then heated for exactly 3 minutes in a boiling water-bath and colours read as quickly as possible, i.e. while still warm, at 625 mμ on the Unicam S.P. 500 spectrophotometer.

3) Culture experiments

Experiment I (using BCM).

Out of 100 snails infected as described above, and maintained at 20°C, 82 survived to the 30th day after infection when the snails were dissected. 15 snails were set aside and dissected under BCM in non-sterile conditions to provide rediae for zero time measurements. Sterile rediae were obtained as described above and the level of infection was high enough to set up 10 cultures each containing 20 to 25 of the largest rediae. During pipetting procedures, numbers of smaller rediae were introduced to the cultures by chance. Culture vessels were 4 inch diameter sterile disposable plastic petri dishes containing 20 ml BCM. The cultures were held at 20°C in a cooled incubator and examined daily for redial activity and cercarial emergence and, where possible, rediae were taken for quantitative measurements.

Experiment II (using BCM)

Out of 100 infected snails maintained at 18°C, 85 survived to the 25th day after infection when the snails were dissected. The earlier date of dissection and lower maintainance temperature were gauged to provide rediae in an earlier state of development to those used in Experiment I (see results). Unfortunately the experiment was handicapped by the fact that the largest rediae obtained were producing daughter rediae and those containing undifferentiated germ balls were very small. It was hoped here to culture rediae with relatively undifferentiated germ balls and the small size of the rediae upset predictions as to the amount of material available for quantitative work. Because of this and the poor results from Experiment I (see results) carbohydrate measurements were abandoned. Nevertheless far more quantitative work was possible than in Experiment I, and groups of rediae were taken at zero time, 24, 72 and 120 hours for dry weight and total nitrogen measurements. 12 cultures were set up as described for Experiment I, but with more careful pipetting to introduce 30 rediae to each culture. The cultures were maintained at 20°C in the cooled incubator.

Experiment III (using supplemented BCM)

In an attempt to provide a more solid environment and/or proteins

and growth factors absent from BCM, cultures were set up in 1.) BCM with 5% gelatin. 2.) BCM with 20% whole Lymnaea stagnalis haemolymph. 3.) BCM with both 5% gelatin and 20% whole Lymnaea stagnalis haemolymph. The haemolymph was obtained by puncture of the shell spire of large Lymnaea stagnalis from a local wild population. Only uninfected snails were used, and each provided 1 to 2 ml of haemolymph when fully drained. Thus enough haemolymph could be obtained in the future for extraction and partial purification of lipid and protein supplements for addition to BCM. Haemolymph was collected just before setting up the cultures and was stored in ice-cooled vessels. All media were sterilised by candle filtration. Three cultures were set up for each medium in 4 inch sterile disposable plastic petri dishes, each containing 10 sterile rediae dissected from snails 25 days after infection. Cultures were maintained at 20°C in the cooled incubator. Attempts to set up further cultures with a BCM overlay over precipitated collagen (Sigma) were unsuccessful. Attempts to precipitate collagen from solutions in 0.2% and 0.02% acetic acid produced no layers suitable for culture work.

Results

(1) The techniques described for obtaining sterile rediae were not altogether satisfactory. Not only did the dissections take a very

long time, but also the sterility of the rediae obtained was questionable: as shown by acute contamination of some subsequent cultures. As rediae are covered with surface mucus, any contamination with micro-organisms during dissection from the snail, would be almost impossible to remove. The alcoholic sterilisation of the snails did not affect the rediae in any way. A good level of infection was always found with over 90% of snails providing 4 or 5 'large' rediae (at least 20 µg dry weight) and numerous smaller rediae, by the 30th day after infection.

(2) Techniques for dry weight measurement and determination of total Nitrogen were satisfactory. The weights of 'blank' foil pieces were not significantly changed by the heat treatment, and the presence of Aluminium and additional procedures in colour development did not increase errors. Measurements of redial size were of little value, and it was hoped at this stage that increases in dry weight and total Nitrogen in the cultured rediae would be large enough to be demonstrable above the wide variation in size (see Experiment II below). The difficulties described with the anthrone method for determination of total carbohydrate were not unexpected. Glick (1963) cites the "capricious nature" of this reaction. Table 12 gives the optical densities recorded using the successfully modified method. The success of the modified method was attributed to a.) The omission of an ethyl acetate phase and therefore easier standardisation

of procedure. b.) The presence of a high concentration of warm H_2SO_4 after final colour development, preventing turbidity. Empirical trials showed that turbidity occurs whenever a solution of anthrone in concentrated H_2SO_4 is significantly diluted with a cold aqueous phase.

Table 12.

µg Glucose in aliquot	Optical densities (Triplicates)		
Blank	0.000,	0.005,	0.008
36	0.243,	0.243,	0.241
72	0.512,	0.498,	0.486
108	0.790,	0.824,	0.795

(3) Experiment I

The experiment was almost a total failure with very high mortality of rediae and contamination of cultures. During the first 72 hours, 6 cultures were discarded showing massive contamination with bacteria, fungi, and protozoa. Contamination was obvious, without resort to sterility tests. The loss of material restricted quantitative work to zero time and 24 hour cultured rediae.only. The results (Table 13) have no significance as culture criteria, but do show the rough composition of rediae. Comparing the dry weights with those recorded by Sommerville

(Appendix II) for 30 day-old rediae it can be seen that the rediae dissected here were very large indeed.

After setting up the 10 cultures, examination showed the large rediae to be in good condition, actively moving and containing recognisable cercariae with head/tail differentiation, some of which were moving inside the rediae. These immature cercariae showed no pigmentation. Also seen were smaller rediae containing undifferentiated germ balls and some immature cercariae moving free in the medium. It was thought that the latter resulted from rediae damaged during snail dissection, and were introduced by chance during pipetting procedures. It was unfortunate that no counts or measurements were made on these smaller rediae and free cercariae because they went on to provide the most interesting aspect of the experiment.

Examination of the cultures at 24 hours showed that the majority of the large rediae had become inactive and very swollen. Sufficient active rediae of normal appearance were taken for quantitative measurements, allowing one determination each for total nitrogen and total carbohydrate. Immature cercariae continued to move inside the bodies of swollen rediae, but the numbers of cercariae free in the medium appeared to have increased. These continued to show active movement. The smaller rediae in the cultures remained in good condition. Neither the large nor smaller rediae nor free cercariae attached themselves

to the plastic culture vessel as did the sporocysts (Part V).

At 48 hours, all the large rediae appeared to be dead. They were totally inactive and very swollen and opaque. Redial tissue appeared to have broken down and the gut was indistinguishable. At this stage both immature and pigmented cercariae were seen moving inside the bodies of the dead rediae. Plate 31. shows the appearance of dead and dying large rediae, and of the smaller rediae which remained active and in good condition. The most surprising development was the appearance of large numbers of apparently fully mature pigmented cercariae free in the medium. These had fully-developed long tails and some were observed forcing their way out of the bodies of dead rediae, tail first. The free cercariae showed continuous motion through the medium with tail movements and body flexure.

With 6 cultures lost through contamination, and 2 more used up for quantitative measurements, only 2 cultures remained for observation of the cercariae and smaller rediae. At 50 hours, 5 of the most mature cercariae, selected for long tails and pigmentation, were removed from culture and placed in distilled water in solid watch-glasses, then observed for 60 minutes. All showed very agitated movement in contrast to the slow movement seen while in BCM. Despite very rapid tail thrashing, no tail-shedding or encystment was observed.

The procedure was repeated at 24 hour intervals with identical results until at 98 hours 1 out of 5 cercariae shed it's tail and encysted, after 5 minutes in distilled water. At 122 hours all 5 cercariae encysted within 5 minutes, and 3 repeat experiments confirmed this 100% result. At this stage there were still large numbers of immature cercariae in apparently good condition in the cultures.

The cultures were terminated at 125 hours. At this time, the small rediae in the cultures were still very active and apparently healthy, but with no original counts or size measurements it was pointless to continue the experiment for the sake of survival records. No dead cercariae were seen, and no encystment occurred in the cultures. On terminating the cultures, dissection of the dead large rediae released more cercariae at all stages of development, all still alive and active.

Table 13. Summary of measurements for Experiment I. ('N ' and 'CHO' indicate the samples used for nitrogen and carbohydrate determinations).

1) Dry Weights

Time	Sample No.	No of rediae	Size date (mm)*	Total dry weight (µg)	Mean dry weight (µg)
0	1 (CHO)	20	1.88, 0.14 (0.42, 0.034)	352.0	17.6
	2 (CHO)	20	1.91, 0.14 (0.41, 0.034)	447.6	22.4
	3 (N)	20	1.64, 0.13 (0.39, 0.037)	359.7	17.9
	4 (N)	16	1.55, 0.13 (0.32, 0.025)	319.4	20.0
24 hrs	1 (N)	15	1.60, 0.15 (0.36, 0.075)	251.2	16.8
	2 (CHO)	15	1.74, 0.12 (0.46, 0.011)	343.8	22.9

2) Chemical measurements

a) Nitrogen

Time/Sample No.	Total Nitrogen (µg)	Mean Nitrogen per rediae (µg)	Nitrogen per µg sample
0/3	51.71	2.59	0.144
0/4	50.58	3.16	0.158
24hrs/1	37.64	2.51	0.150

b) Carbohydrate

Time/Sample No.	Total Carbohydrate+ (µg)	Mean Carbohydrate per rediae (µg)	Carbohydrate per per µg sample
0/1	6.52	0.33	0.019
0/2	6.84	0.34	0.015
24 hrs./2	3.88	0.26	0.011

* Size data are: Mean length, mean breadth (standard deviation length, standard deviation breadth).

+ Optical densities were very low i.e. 0.025 to 0.044, compared with 0.463 for a 72 µg standard.

The figures show the variability of the material and the difficulty in grouping rediae by size measurements. The results suggest that the criteria developed are probably inadequate to show changes in cultured rediae unless sample groups are very carefully selected from a huge population. The carbohydrate content of rediae appears very low, but it can be estimated from the total Nitrogen figures that the samples are mainly protein, with very little weight of carbohydrate, lipid etc. e.g. Time, 0, sample 3 is estimated to contain approximately 320 µg protein. From the results it can be seen that the 0.5 ml aliquots used in carbohydrate determinations contained less than the "5 to 200 µg of carbohydrate" recommended for the anthrone method (Kahan, 1953).

Experiment II

Contamination of cultures was again serious, and 4 cultures were discarded after 72 hours. Dissection of the snails 25 days after infection did not give the results hoped for, as the largest rediae contained developing daughter rediae. The rediae selected for dry weight and total nitrogen determinations were much smaller, containing undifferentiated germ balls. Rediae after 24 hours in culture appeared very active and no mortalities were seen in any cultures. Subsequent mortalities and the results of the quantitative measurements are given in Table 14. At 72 and 120 hours, the rediae appeared less healthy and their movements became sluggish together with some opacity of

the body wall. No differentiation of germ balls was observed over the 5 day culture and again no rediae attached to the culture vessels.

Table 14. (Summary of measurements for ExperimentII)

Time	Sample No.	No. of Rediae	Size (mm)*	Dry Wt. (µg)	Mean Dry Wt. (µg)	Total Nitrogen (µg)	Nitrogen per rediae (µg)	Nitrogen per (µg)	Mortality
0	1	22	0.81,0.17(0.18,0010)	89.0	4.05	7.72	0.351	0.087	-
0	2	15	0.84,0.14(0.15,0.030)	56.0	3.73	4.17	0.278	0.075	-
24hrs.1		15	0.75,0.14(0.12,0,037)	64.0	4.27	6.39	0.426	0.100	0
24hrs.2		15	0.72,0.15(0.08,0.030)	83.4	5.56	8.85	0.590	0.106	0
72hrs.1		15	0.87,0.15(0.18,0.031)	75.4	5.03	7.83	0.522	0.104	0
72hrs.2		15	0.80,0.14(0.11,0.016)	79.0	5.27	10.33	0.689	0.131	1
120hrs.1		15	0.91,0.14(0.14,0.026)	93.4	6.23	7.93	0.529	0.085	8
120hrs.2		15	0.97,0.14(0.14,0.017)	97.2	6.48	10.03	0.669	0.103	9

Analysis of data

For the dry weights, the regression coefficient with time = 0.436, and a test for significance of this slope gave Student's 't' = 0.076, which is not significant. Thus although the results appear to show a tendency for an increase in dry weight over the culture period, this is not statistically significant. The nitrogen measurements appear meaningless, with a peak at 72 hours. Size measurements again show the difficulty in measuring motile rediae and are not accurate enough to allow estimations of "redial volume".

*Size measurements are again mean length, mean breadth (standard deviation length, standard deviation breadth), and 'mortality' refers to the total number of rediae dead in the sample (a complete culture was sacrificed to provide each sample).

The general result was that these smaller rediae, which were possibly destined to produce daughter rediae in the snail, survived in BCM over a 5-day period with some deterioration and mortalities towards the end, and showed no significant growth or development. It is apparent that BCM cannot support the large increase in growth seen "in vivo" between the 25th and 30th day after infection (see Appendix II), and without stringent selection of rediae for "in vitro" culture from a huge population it will probably be impossible to demonstrate any significant growth in BCM, as the material is so variable.

Experiment III

All the cultures using supplemented BCM were total failures. All the rediae in the gelatin-supplemented cultures were dead and of swollen appearance after 24 hours. Those in cultures supplemented with haemolymph fared slighly better but appeared much less healthy than the rediae in Experiment II with BCM alone, and the cultures were terminated at 72 hours with almost total mortality.

Discussion

Despite the failure of the culture attempts in producing growth and development comparable to that "in vivo" the work did raise some interesting points. The first, already apparent in the descriptions of snail dissections above, and mentioned by Sommerville (see Appendix II), is that around 25 days after infection and thereafter, rediae in

all states of development can be dissected from the snails. Thus rediae for culture must be selected from a vast range of rediae of different shapes and sizes, and with germ balls in different states of development. The criteria used for assessing possible growth and development "in vitro" appeared rather inadequate, mainly because of this variability of material. It is difficult to suggest how more accurate quantitative measurements could be made on cultured rediae, and the principle adopted here of selecting a group of very similar rediae and then sampling the group at intervals, appeared to be the only feasible method. This method could in fact have proved adequate, had more significant growth and development been achieved in BCM. Undoubtedly the development of methods sensitive enough for measurement of individual rediae would ease the problem of criteria and allow enough data to be collected for a meaningful statistical analysis.

In addition to this, the level of infection, the size and state of development of the largest rediae, and the course of infection (for example the production of daughter rediae in Experiment II), appeared to be difficult to control. The size and rate of growth of individual rediae also probably depends on the nutritional value of the sites it can find free in the digestive gland and thus even rediae with germ balls in exactly the same state of development can show great variation in size and shape. Just about the only

thing that can be firmly predicted with experimental infections is the earliest date at which cercariae could emerge, and even this will depend on the environment and food supply of the snails (Kendall and Ollerenshaw, 1963). All this means that even with strict temperature and humidity control and a good food supply, rediae obtained by dissection from snails are likely to be very variable. It is an interesting possibility that rediae cultured from sporocysts "in vitro" could provide a far more uniform source of material for further study.

The incidental observations on germ balls and cercariae in Experiment I show that BCM is a reasonable substitute for haemolymph and for redial body fluids. The poor culture results as far as the whole rediae were concerned suggests that rediae need to use their gut and may need solid food and a semi-solid environment. The developing germ balls, however, must receive their nutrients in solution and the rather sparse observations here suggest that BCM could be used to culture germ balls "in vitro", perhaps up to mature cercariae. Indeed the "in vitro" culture of cercariae up to a stage at which they would encyst could be said to be the only positive result in this section of the work. Even here, however, the development may have been made possible either by using endogenous reserves, or by using nutrients derived from the broken down tissues of the dead rediae. It seems

unlikely that this breakdown of redial tissue and the 'tail-first' emergency of cercariae ever have any parallel "in vivo". It is good circumstancial evidence for the physical suitability of BCM that apparently fully mature cercariae will not encyst in the medium, but require transfer to water alone: an osmotic change similar to that seen "in vivo".

In general, it seems that BCM is inadequate for the successful "in vitro" culture of rediae. The good survival of the smaller rediae in Experiments I and II is difficult to explain, but it may be that these younger rediae can survive well by absorbing nutrients through the body surface and that intake of solid food and use of the gut is only essential to the larger rediae to support the synthesis of tissue in the latter stages of cercarial production. Thus the smaller rediae may have survived in BCM as they may do during the migratory phase to the digestive gland "in vivo". It is difficult to suggest how a fully satisfactory culture medium could be developed. The points made when discussing the sporocyst cultures (Part V, discussion) with respect to pO_2, lipids, and proteins all apply equally in the case of rediae. What may be required is an exhaustive analysis of digestive gland tissue and, perhaps, the abandonment of the idea of a fully defined medium. If large rediae do require solid food this could then be added either as an artificial substitute, or by actually growing a

culture of mollusc tissue. Chernin (1963, 1964) has done useful ground work towards cultures of this type. In such a system, BCM could be used as a liquid overlay. On the question of sterility, the contamination of cultures in the present study was a very serious problem. While this may have been due to suspect techniques during dissections, the possibility that the digestive gland is <u>not</u> a sterile site cannot be discounted.

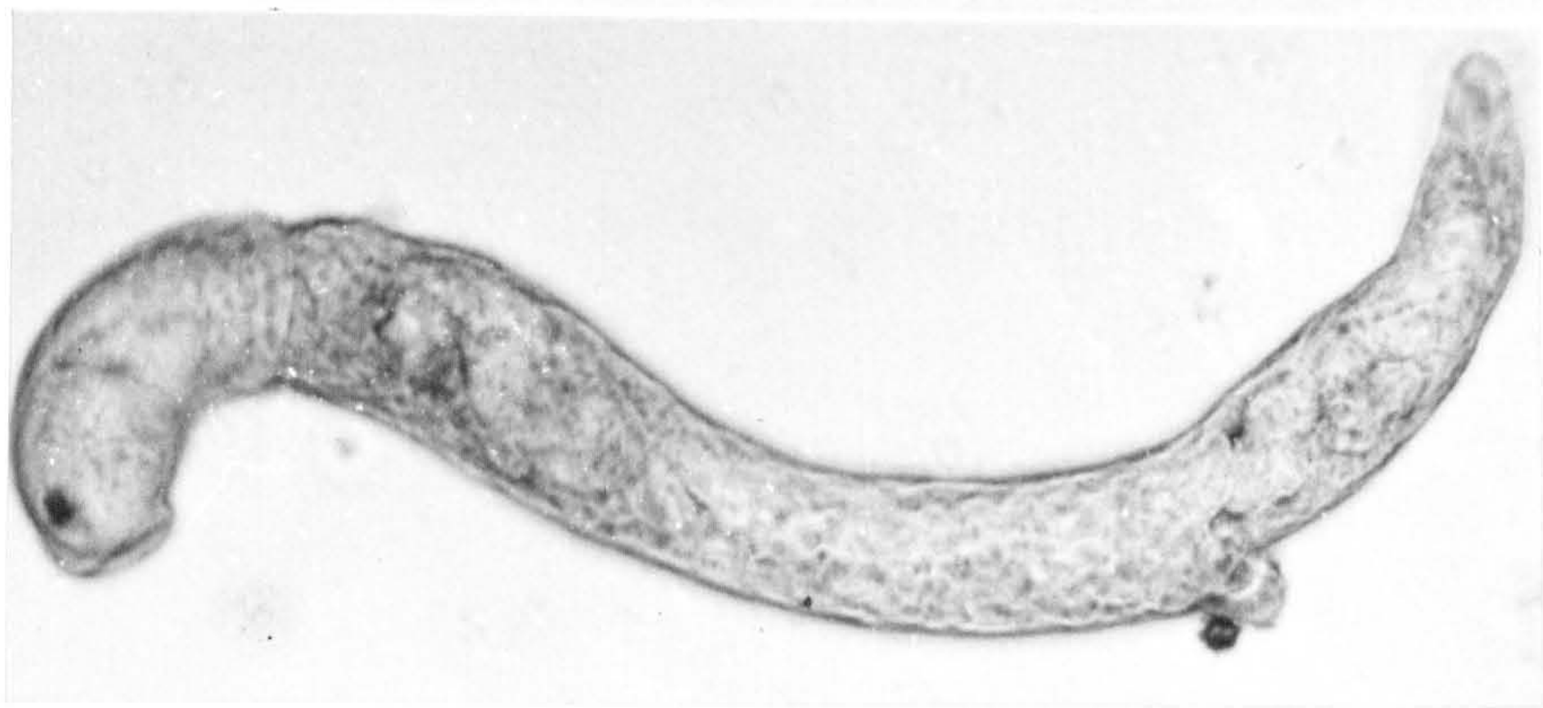
Plate 31. Rediae after 48 hours culture in BCM.

a.) Healthy, active, small redia.

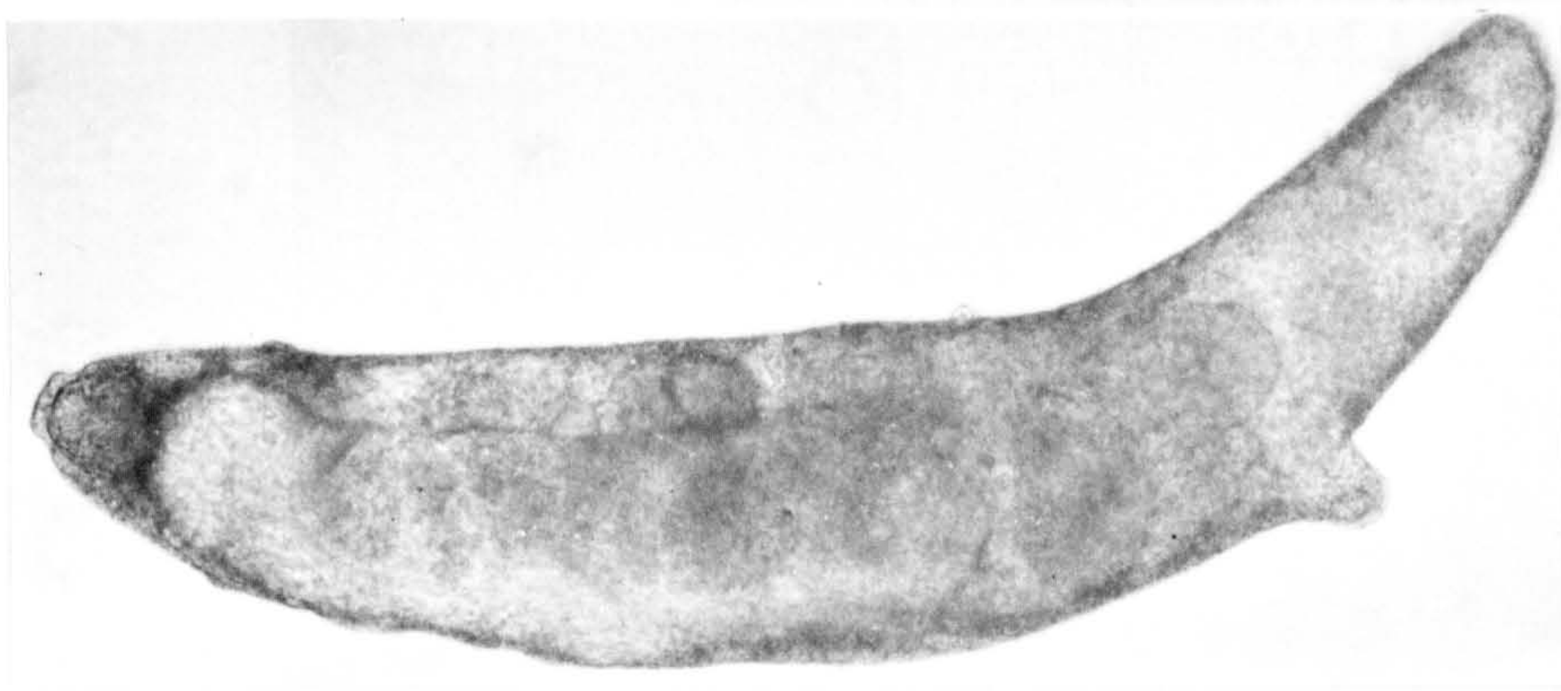
b.) Large redia; sluggish and inactive with the body wall turning opaque.

c.) Dead, large redia with all internal structure broken down.

a)



b)



c)

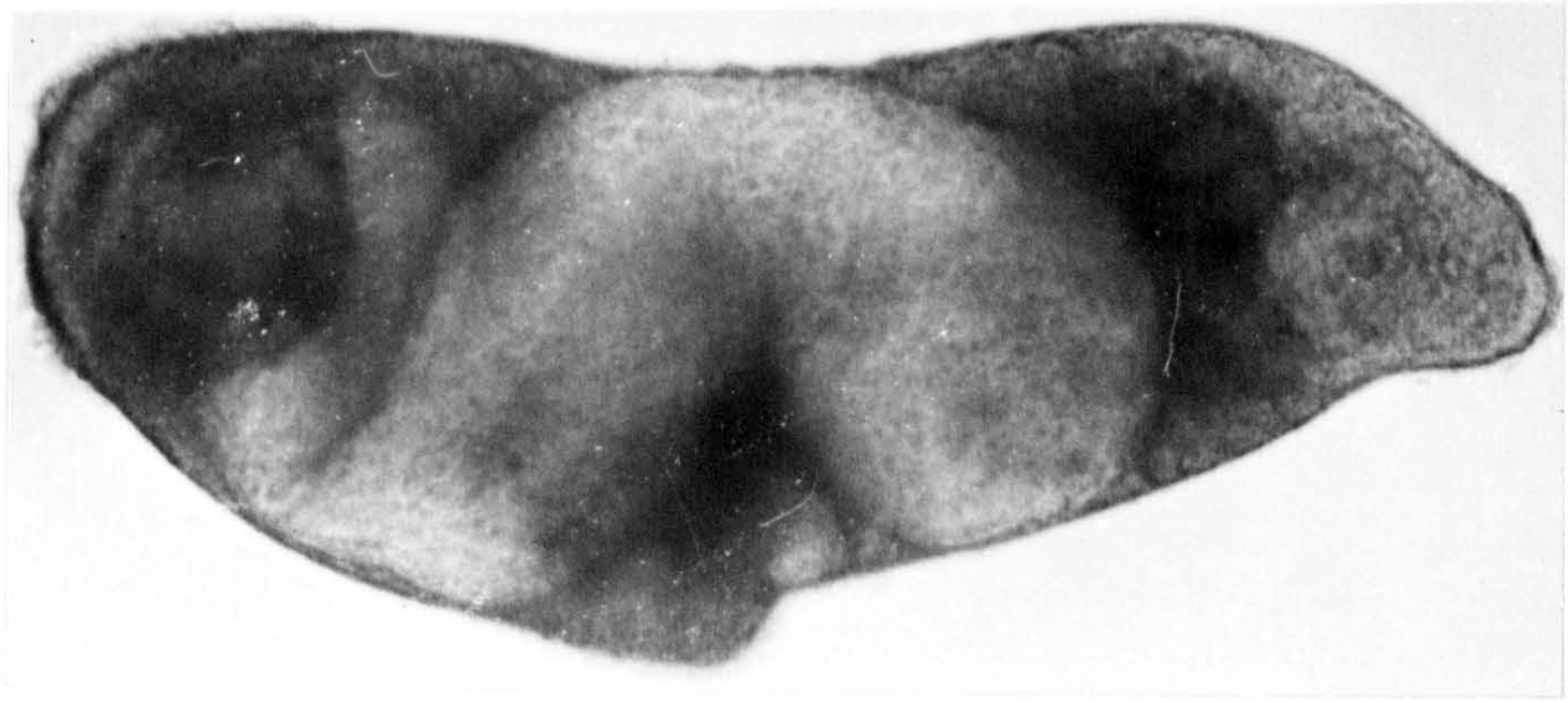


PLATE 31

SUMMARY AND CONCLUSIONS

The haemolymph of Lymnaea truncatula is similar in composition to that of other closely related molluscs. Analysis of the major chemical constituents of the haemolymph gives sufficient information to formulate a useful physiological saline, and empirical addition of other compounds provides an artificial haemolymph which can be used for " in vitro" culture work with larval Fasciola hepatica. The medium BCM developed here, however, cannot support normal growth and development of sporocysts and rediae "in vitro". BCM does stimulate miracidia in batch cultures to shed their epidermal cells and to undergo a limited development as young sporocysts. Also it appears that immature cercariae can complete their maturation"in vitro" in BCM.

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Appendix I - The pO_2 of haemolymph

1) Apparatus and calibration.

A Beckman micro-oxygen electrode was used, connected to a Radiometer pH meter (PHM 27) fitted with a gas monitor attachment (PHA 927). The apparatus was connected to a 100 m V external recorder (Specdomax W) with a 50,000 Ω potentiometer on the input side to adjust the deflection and base lines. Chart speed was kept constant at about 2.5 cm per minute.

The principle of calibration was to assume that water in equilibrium with atmospheric oxygen is at 160 mm of Hg pO_2. Thus one standard reference point was obtained by bubbling air from a small pump through distilled water. This was normally performed in a 50 ml conical flask with a rubber bung bored to admit the 'gassing' capillary tube and the oxygen electrode. Both of these were kept as loose fits in the bung to allow escape of gas. Water was aerated for at least 30 minutes with fast constant bubbling. After this time, the pH meter and recorder were adjusted so that the recorder trace read 100%. In practice, the pO_2 range knob was kept at the 80-220 setting and the pO_2 span control turned to its full clockwise setting. The pH meter was then found to read between 13.5 and 14.0 and the recorder was set using the intermediate potentiometer for coarse adjustment and the central pO_2

span control for fine adjustment. It should be stressed that as the oxygen electrode was a Beckman product not designed for use with this instrument, the above settings were purely arbitrary and were simply the best settings found for obtaining a sensitive response at the recorder to any change in pO_2. Thus the pH meter was used merely as a voltmeter and the gas monitor was never used for direct readings. The steady trace adjusted to read 100% was taken to represent 160 mm of Hg. pO_2 . It was important to position the 'gassing' capillary tube so that no gas bubbles passed near the membrane of the oxygen electrode. Small gas bubbles readily stuck to the membrane and caused abnormal readings (see later "air bubble effects" Figures 13. to 15.). Usually a steady trace was obtained without difficulty and any "noise" on the trace indicated that the electrode membrane was faulty. New membranes were left, with the apparatus switched on, for several hours after installation, before attempting a calibration.

Several methods were tried for producing the second required standard reference point i.e. zero pO_2. The addition of a suspension of yeast caused the recorder trace to drop to a steady reading of around 20% after about 5 minutes, but here the calibration solution had to be stirred continuously to produce a smooth curve and a fresh solution used to check the response of the apparatus back to

100% reading at 160 mm of Hg. pO_2. Using 5% glucose instead of distilled water as the calibration solution, a similar calibration was achieved using the Beckman glucose oxidase solution, supplied with the electrode. Here, the recorder reading dropped to 19.4% to represent zero pO_2, when 10 drops of the enzyme preparation were added to 25 ml aerated 5% glucose. The most convenient method of achieving zero pO_2, however, was by the use of nitrogen. When nitrogen is fed to the "gassing" capillary instead of air the recorder trace drops sharply and steadily until an equilibrium representing zero pO_2 is reached after about 15 minutes. The nitrogen was always bubbled fast to stir the solution. It was found that the preparation of 2 flasks, one "gassed" with air and the other with nitrogen, provided an elegant method of checking the response of the electrode and adjusting the zero and f.s.d. lines while using the electrode. Figure 9. shows the excellent calibration obtainable by this method, and shows that with practice in assembling the electrode and adjusting the instruments, over 90% of the chart width could regularly be used. A fresh calibration was made before every determination and each new membrane calibrated several times.

2) Attempts to read the pO_2 of haemolymph of Lymnaea truncatula.

Lymnaea truncatula is too small for insertion of the oxygen electrode in the steel hypodermic supplied by Beckman. Thus glass

sheaths were made to surround the electrode, by drawing out 100 µl capacity Shandon microcapillaries, hoping that enough haemolymph would rush up the tapered section of the glass sheath to surround the electrode. The glass sheath and enclosed electrode were mounted on a micromanipulator and inserted into snails anaesthetised using Ether and Carbon Dioxide, as described for haemolymph collection (Part II). The method was fraught with difficulties. Apart from the fact that there was not really enough haemolymph to fill the glass sheath and surround the electrode, problems were caused by air bubbles and mucus swept in with the haemolymph. Both of these caused trouble at the membrane; and membranes contaminated with mucus were discarded. Also it was felt that atmospheric oxygen was contaminating the sample very rapidly. The plasticity of snail tissue and the presence of mucus made controlled insertion of the glass sheath and the enclosed electrode almost impossible. Further attempts were made to pool haemolymph from several snails under oil, but these met with similar difficulties, particularly the contamination of the membrane with oil. It was obvious that even if the methods could be perfected, anaesthetised snails would have to be used, and these would not give any idea of the pO_2 of normal haemolymph. Thus all attempts to determine the pO_2 of Lymnaea truncatula haemolymph were abandoned.

3) The determination of the pO_2 of Lymnaea stagnalis haemolymph

Lymnaea stagnalis is a large aquatic pulmonate with a relatively

large haemolymph volume. It was found that the technique of enclosing the electrode in a glass sheath, drawn out at one end for easy penetration of snail tissue, worked well with this larger species. The glass sheath - a 100 μl Shandon microcapillary - was inserted into the visceral haemocoele of the snail as rapidly as possible. The snails were not anaesthetised for this procedure and were simply taken from the water; blotted dry; and hand-held. After insertion of the glass, the snail's head-foot contracted and haemolymph began to flow up the glass tube and around the electrode. To allow easy flow, the electrode stem was a fairly loose fit in the glass sheath, and haemolymph overflowed from its open end. The flow of haemolymph was easily observed and frequently lasted for several minutes. Thus the pO_2 of haemolymph could be read without fear of contamination from atmospheric oxygen. If the flow of haemolymph past the electrode was not maintained, however, the recorded pO_2 was seen to drop significantly. This was because the electrode was using up a significant amount of oxygen in a small stagnant volume. Figure 10. shows this effect of depletion of oxygen; and Figures 11. and 12. show normal traces with continuous haemolymph flow.

Most of the difficulties during determinations came from incorrect insertion of the glass sheath (termed"probe" in the Figures). If the probe became dislodged during a determination or became a very

loose fit in the snail tissue, air bubbles were often swept in with the haemolymph and caused abnormal peaks as they passed the electrode membrane (Figures 13. to 15.). As can be seen in Figure 13., this did not always ruin the determination. Other difficulties resulted from inserting the probe too slowly. Figure 14. shows that water trapped under the shell could fill the glass sheath. This "shell water" was forced out over the head-foot as the snail contracted and, if the probe had not been fully inserted, it ran in to give a pO_2 reading much higher than that of haemolymph and then stagnated giving an oxygen depletion effect (Figure 14.). Figure 15. shows the effect of the electrode membrane touching the glass sheath. This was rarely a problem but could occur if the probe was jolted and the electrode slipped down the glass sheath to bear on the taper. Mucus contamination of the membrane occurred on very few occasions, but when it did very low pO_2's were recorded (Figure 16.). Mucus contamination was usually demonstrated by the failure to obtain an f.s.d. when the electrode was transferred from the glass sheath to water at 160 mm of Hg. pO_2. (As can be seen from the figures, zero and f.s.d. checks were employed on numerous occasions between snails). In Figure 16., washing the electrode in the aerated water was sufficient to clean off the mucus and give a normal response when reading haemolymph from another snail. However during critical determinations, membranes were always discarded if mucus contamination was suspected.

It can be seen from the normal traces (Figures 11. and 12.) that the equilibrium level ("taken as snail pO_2") shows minor fluctuations after the stabilisation period (B—C). Thus for calculation purposes, the pO_2 was read at every centimetre line on the recorder trace for the first 5 cm after stabilisation (i.e. C—D) and the mean value was taken to represent haemolymph pO_2. Sometimes (see Table I below) the fluctuationswere larger than in Figure 11. and sometimes, as in Figure 12, the trace showed a downwards or upwards slope suggesting oxygen depletion or enrichment. However, the above system for reading the trace was used as long as a flow of haemolymph past the electrode was observed. Table I gives the trace readings of calculated pO_2 for each of 10 snails.

Table I. (100% represents 160 mm of Hg. pO_2, and 5.8% represents zero pO_2.)

Snail/size (cm)		Trace readings (%)					pO_2 (mm of Hg.)	
		1	2	3	4	5	Mean	
1.	(3.7)	24.2	23.5	23.0	22.8	21.0	22.90	29.0
2.	(3.6)	18.7	18.4	18.2	17.8	18.0	18.22	21.1
3.	(3.4)	19.0	16.0	17.0	16.8	16.4	17.04	19.1
4.	(3.4)	16.0	15.6	18.0	15.0	14.6	15.84	17.1
5.	(3.7)	17.7	19.0	16.2	16.5	15.9	17.06	19.1
6.	(3.9)	20.7	21.0	20.8	20.6	20.2	20.66	25.0
7.	(3.6)	19.0	18.5	19.0	19.0	18.9	18.88	21.9
8.	(3.6)	13.2	14.0	13.6	13.0	13.3	13.42	12.6
9.	(3.4)	14.9	13.7	13.8	13.4	12.0	13.56	12.9
10.	(3.5)	28.0	26.8	27.2	25.5	25.5	26.60	35.1

The mean of the 10 determinations is 21.3 mm of Hg. pO_2, but the only significant deduction that can be made from such variable results is that the pO_2 of the haemolymph was very low, and lay between 10 and 40 mm of Hg.

CALIBRATION CURVE

A. – in water; gassed with air for 30 minutes (160mm. of Hg pO_2).

B. – gassed with Nitrogen.

C. – pO_2 falls.

D. – recorder switched off / restarted after 15 minutes.

E. – constant for 10 minutes (taken as zero pO_2).

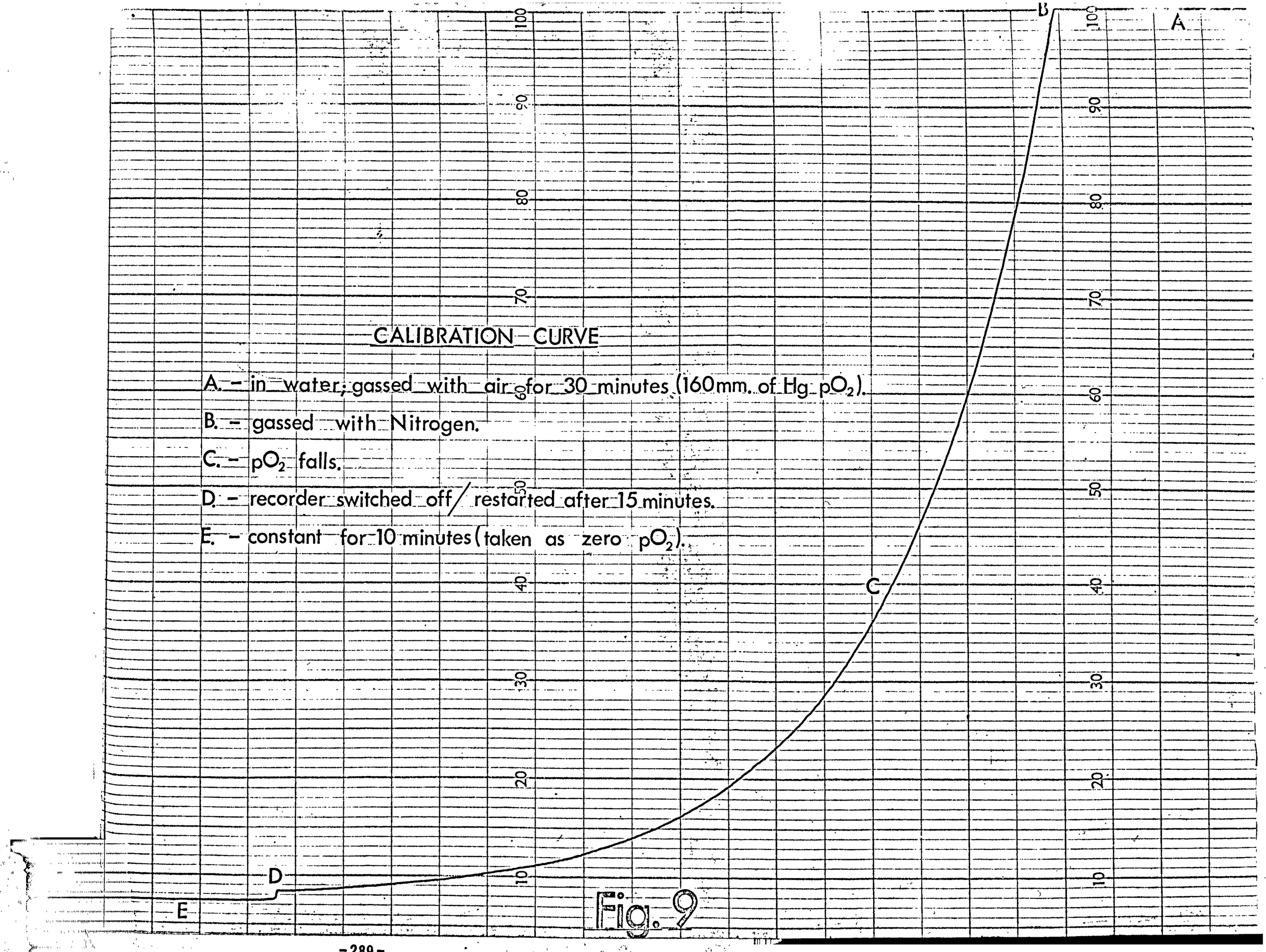

Fig. 9

A. – in water (zero pO_2).

B. – check f.s.d. (160 mm. of Hg pO_2)/air.

C. – probe into snail.

D. – fluctuations as haemolymph flows out.

E. – haemolymph stops flowing.

F. – pO_2 falls after stagnation.

G. – electrode withdrawn.

100
90
80
70
60
50
40
30
20
10

C

HAEMOLYMPH FLOW EFFECT

[6·0 represents zero pO_2
100·2 " 160 mm. of Hg pO_2]

E
D
F
G
B
A

Fig. 10

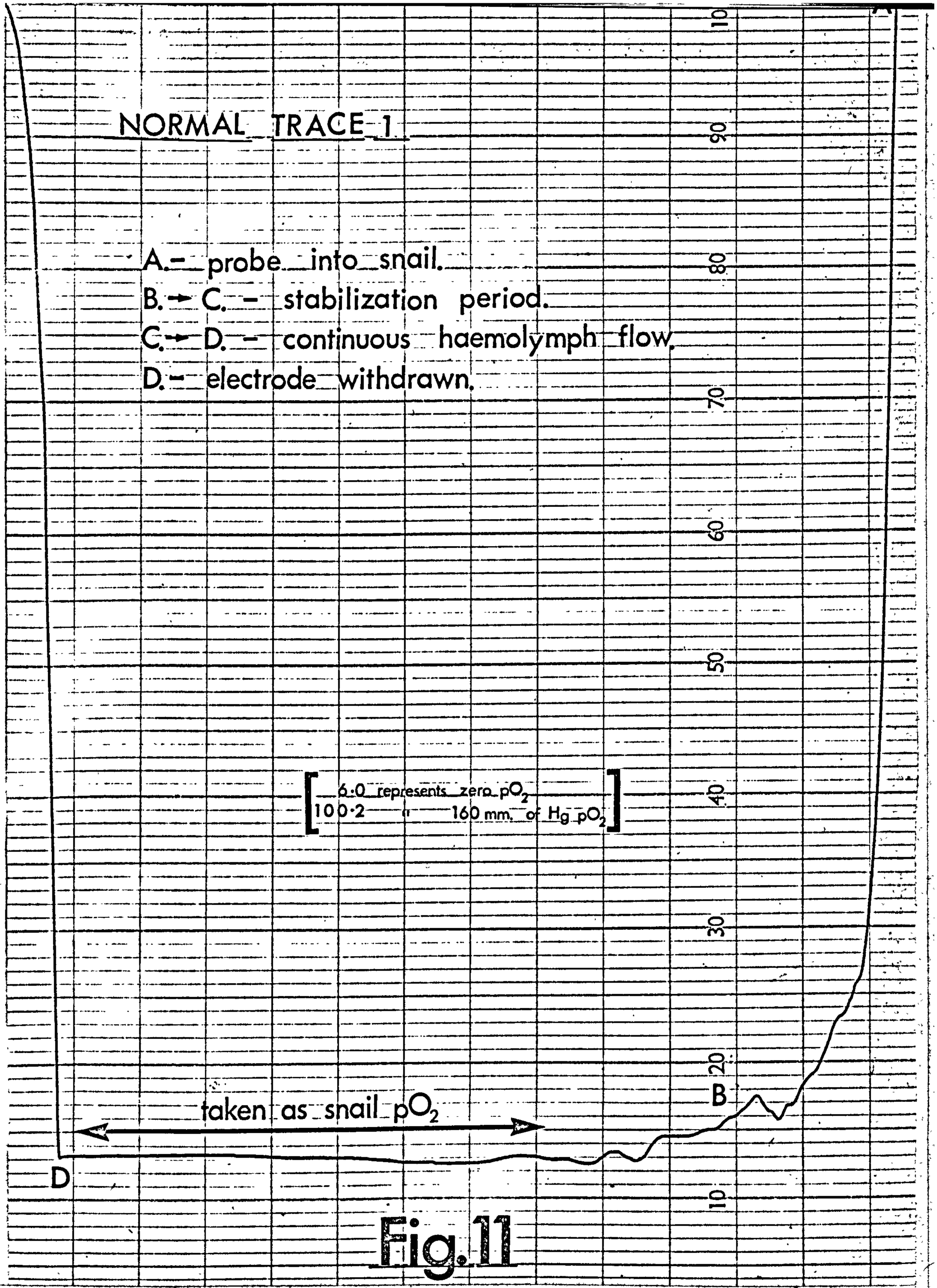
NORMAL TRACE 1
A.– probe into snail.
B.→ C.– stabilization period.
C.→ D.– continuous haemolymph flow.
D.– electrode withdrawn.
[6·0 represents zero pO_2
100·2 " 160 mm. of Hg pO_2]
taken as snail pO_2
A
B
C
D
10
20
30
40
50
60
70
80
90

Fig. 11

NORMAL TRACE 2

A. – check zero.

B. – check f.s.d.

C. – normal response.

D. → E. continuous haemolymph flow.

E. – electrode withdrawn.

[6·0 represents zero pO_2
100·2 " 160 mm. of Hg pO_2]

taken as snail pO_2

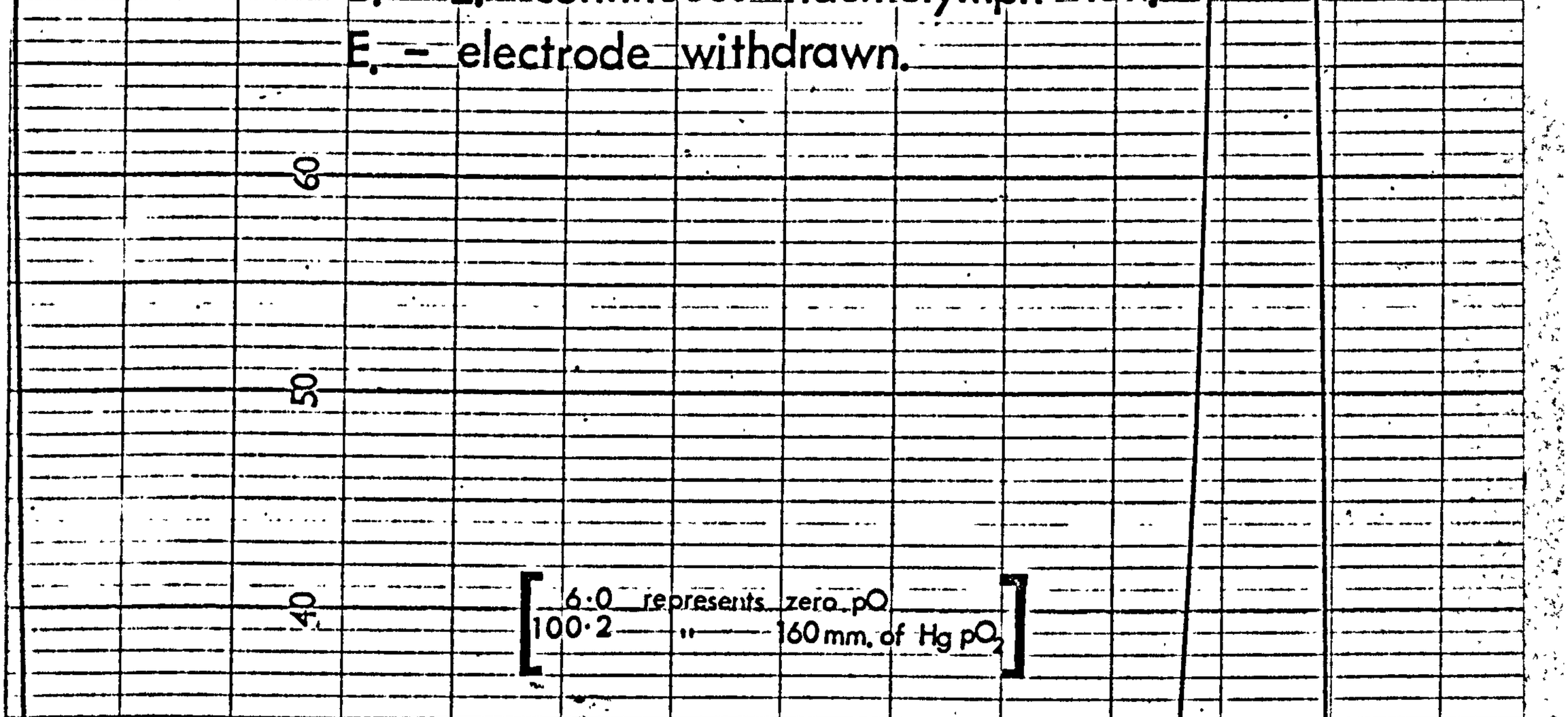

Fig.12

AIR BUBBLE EFFECT

A. – check zero
B. – check f.s.d.
C. – in air
D. – probe into snail
E. – normal response
F. – air bubble passes
G. – normal response
H. – electrode withdrawn

[5·5 represents zero pO_2
100·0 — 160 mm. of Hg pO_2]

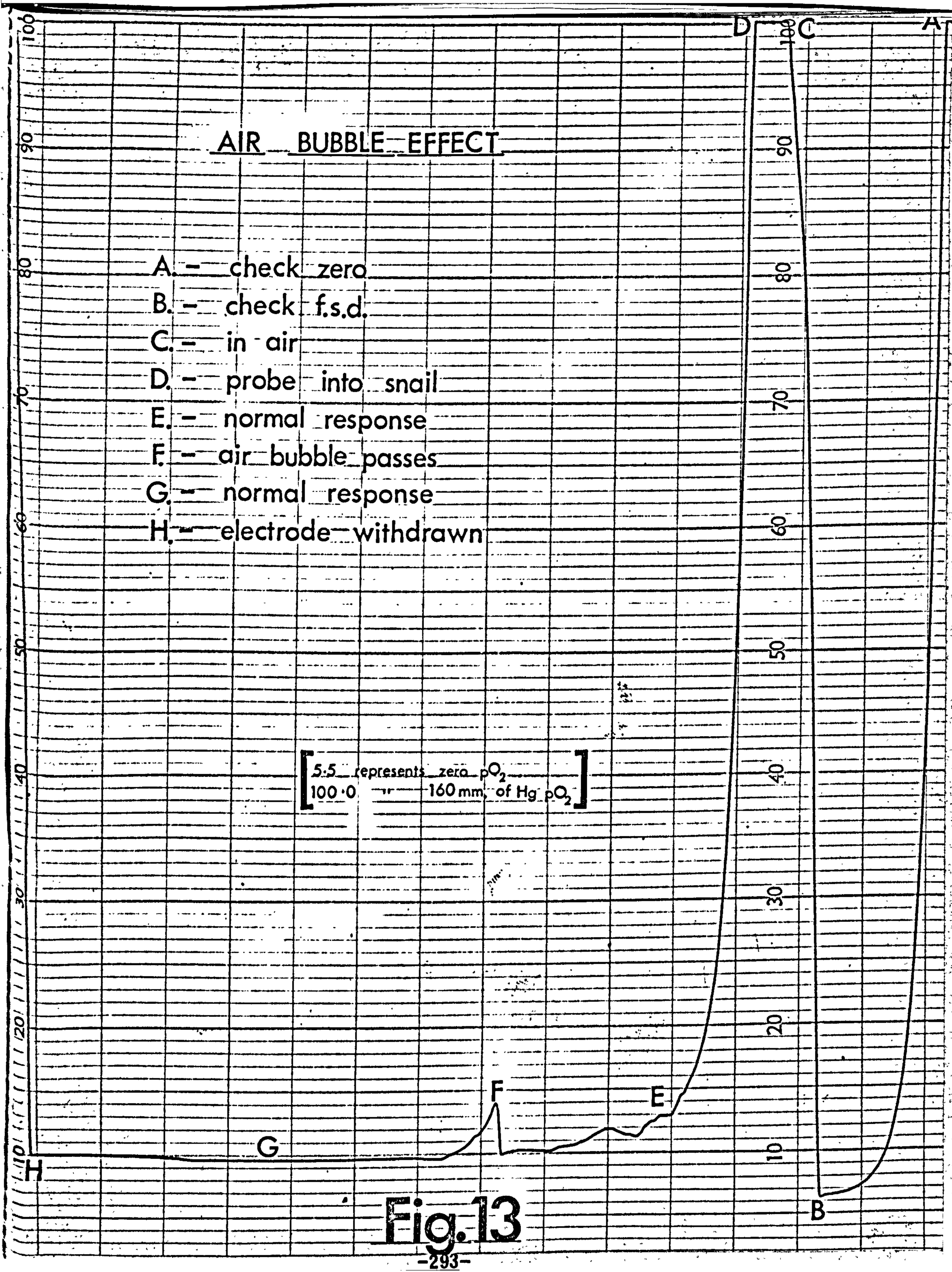

Fig. 13

SHELL WATER EFFECT

A. – check f.s.d.

B. – probe into snail.

C. – air bubble peak.

D. – in stagnant shell water.

E. – electrode withdrawn.

F. – check zero.

[6·0 represents zero pO_2
100·2 " 160 mm. of Hg pO_2]

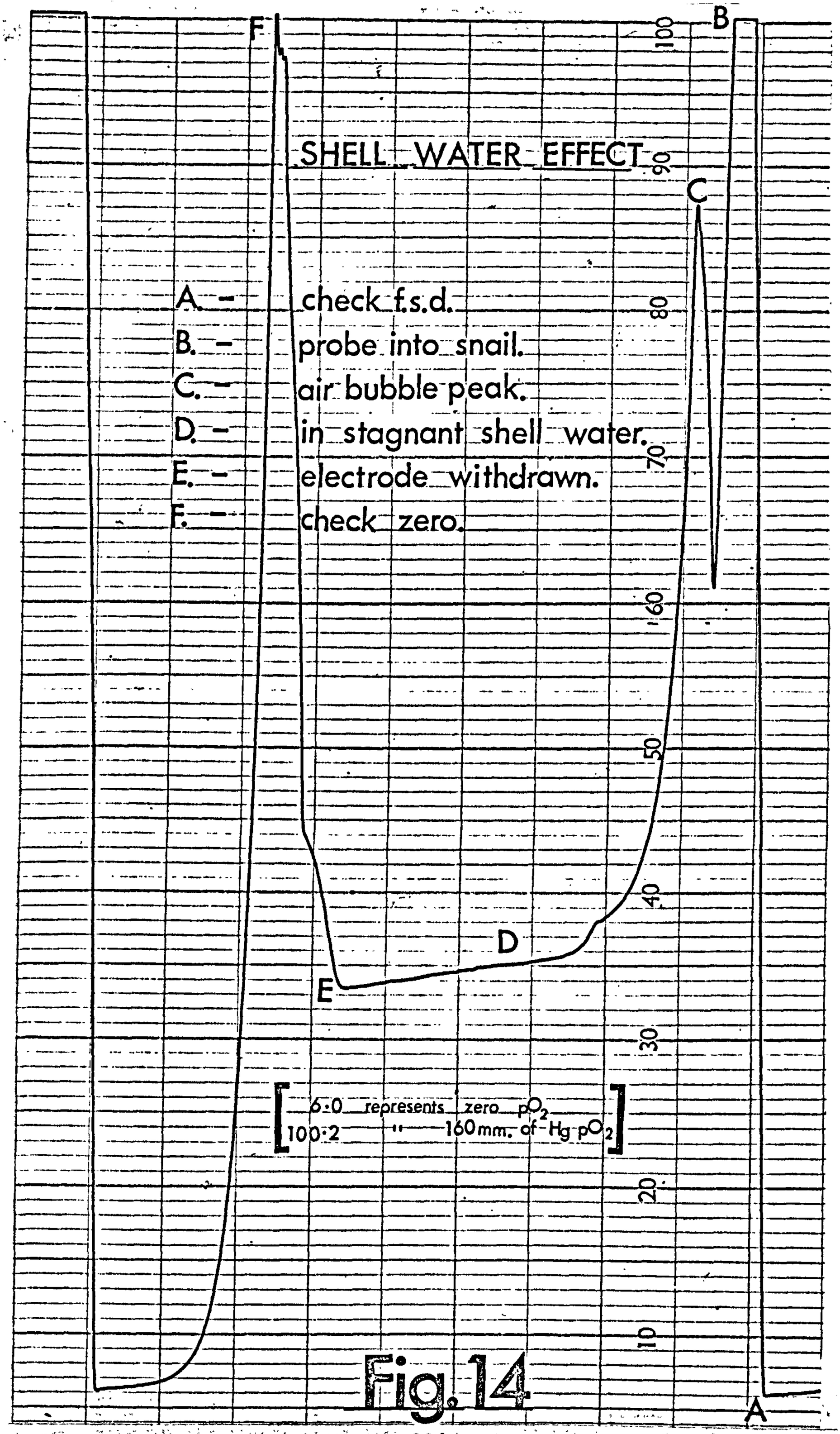

Fig. 14

EFFECT OF TOUCHING THE MEMBRANE

A. – in air./check zero.
B. – check f.s.d.
C. – probe into snail.
D E. – air bubble effects.
F – glass touches membrane.
G – pO_2 falls (stagnant haemolymph).

[6·0 represents zero pO_2
100·2 " 160 mm. of Hg pO_2]

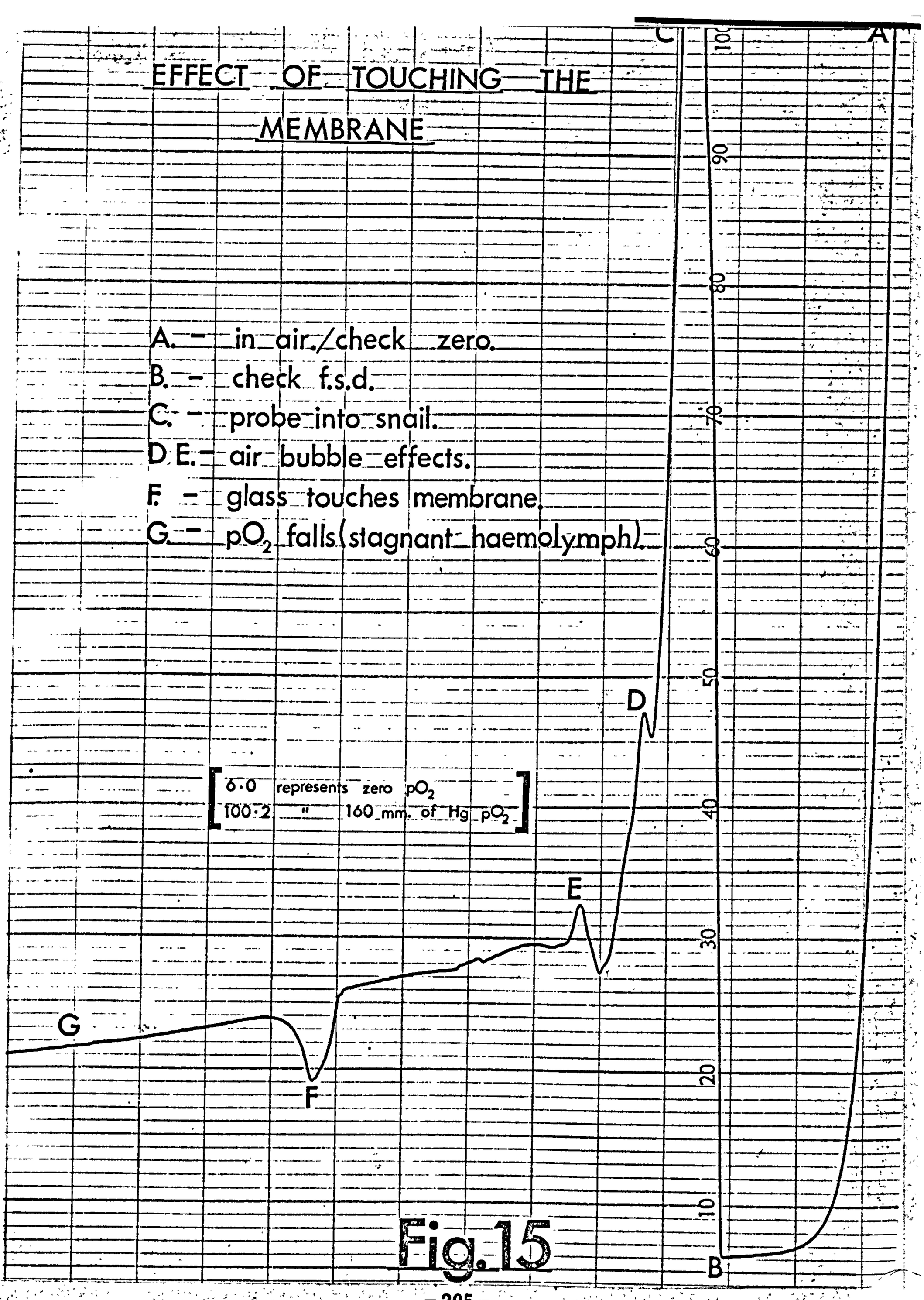

Fig. 15

EFFECT OF MUCUS
A. – very low reading in snail. (check f.s.d.)
B. – mucus prevents f.s.d.
C. – clean probe into snail.
D. – normal response.
[4·5 represents zero pO_2
100·0 " 160 mm. of Hg - pO_2]
100
90
80
70
60
50
40
30
20
10
A
B
C
D

Fig.16

Appendix II. The growth and development of rediae "in vivo" (Sommerville, 1966, personal communication)

Lymnaea truncatula of 2 mm total shell length were infected by exposure to 3 to 5 miracidia each, and maintained on flourishing algal cultures. For each experiment a batch of 200 snails was used. Snails were removed daily from the sides of the dishes to ensure that none went into aestivation. On the 17th day after infection, and at convenient intervals after this, 5 snails were removed and dissected under 0.475% saline (a substitute for the balanced salt solution described in Part IV). The smallest and largest rediae from each snail were taken and collected in 2 solid watch glasses giving 2 groups of 5 rediae - representing the most-developed and least-developed rediae at the stage of the infection. The fresh and dry weights of each group of rediae were then determined. For fresh weights, the rediae were dried with fine strands of tissue paper and weighed on aluminium foil discs. These weighings were probably not very accurate, as the operation was performed without controlled humidity. For dry weights, the rediae were dried overnight at 100°C. All weighing was performed on the Cahn gram-electro-balance. Batches of rediae were weighed up to the 46th day after infection. Table I below gives the dry weights for a typical experiment, and Figure 17. shows the results in graphical form.

Observations on the course of the infection showed that very small

rediae were found in the snails 10 days after infection, and that after 14 days most of these had migrated to the digestive gland. It seems, however, (Figure 17.), that the sporocysts release rediae over a period of many weeks as small rediae are found throughout the course of the infection. The small migrating rediae had small undifferentiated germ balls, and a gut occupying up to two thirds of the body length. The collar and papillae were very prominent on the small rediae, and their guts were filled with rounded colourless cells. It appeared that the gut of the larger maturing rediae did not increase in size as the rediae grew. Also the gut contents of the larger rediae appeared to be acellular, and were yellow in colour, resembling the contents of the digestive gland cells. The collar and papillae of maturing rediae became less prominent as the rediae grew and the germ balls began to differentiate to form daughter rediae or cercariae.

The most interesting observation from the point of view of providing rediae for "in vitro" culture was that after about the 20th day after infection, rediae of all sizes and in all states of development (except of course "ready to release cercariae") could be found. Also, in the maturing rediae in the digestive gland, germ balls in all stages of differentiation were found. Thus the development of rediae and their production of cercariae or daughter rediae was seen to be staggered. Attempts to measure redial volume by length and breadth measurements met with the same difficulties described in part VI.

It was found that, following infection with 3 to 5 miracidia, as many as 50 rediae were produced during the 6 weeks after infection, with cercarial release always starting around the 35th day. Table I below shows the great variation in size of rediae from different snails, even with the controlled conditions and careful selection employed here. Figure 17. combines the results obtained from 3 experiments.

Table I. Records of dry weights from a single experiment.

All weights are in μg, and each figure in the dry weight columns refers to a batch of 5 rediae (different numbers of batches were taken on different days). The "dry weight per redia" can be compared with those recorded in Part VI.

Day	Smallest rediae			Largest rediae		
	Dry weights	Mean	Dry Wt. per redia	Dry Weights	Mean	Dry wt. per redia
17	6.0,5.1	5.6	1.1	15.6	15.6	3.1
19	6.8	6.8	1.4	16.6,17.6	16.9	3.4
21	9.3,5.0	7.2	1.4	16.7,17.0,15.1	16.3	3.3
23	5.9,3.6	4.8	1.0	25.5,29.4	27.5	5.1
28	4.4,7.9	6.2	1.2	72.6,54.7	63.7	12.3
30	5.0,4,7,5,4	5.0	1.0	58.3,53.6,57.4,92.5	65.5	13.1
35	10.0	10.0	2.0	76.6,45.6,182.4,162.7	116.8	23.4
40	9.9	9.9	2.0	90.6,121.8,52.4	88.3	17.7
46	7.8	7.8	1.6	54.5,104.7,61.4,29.4*	62.5	12.30

* empty of cercariae

Figure 17. The dry weights of rediae taken from Lymnaea truncatula during the 6 weeks after infection.

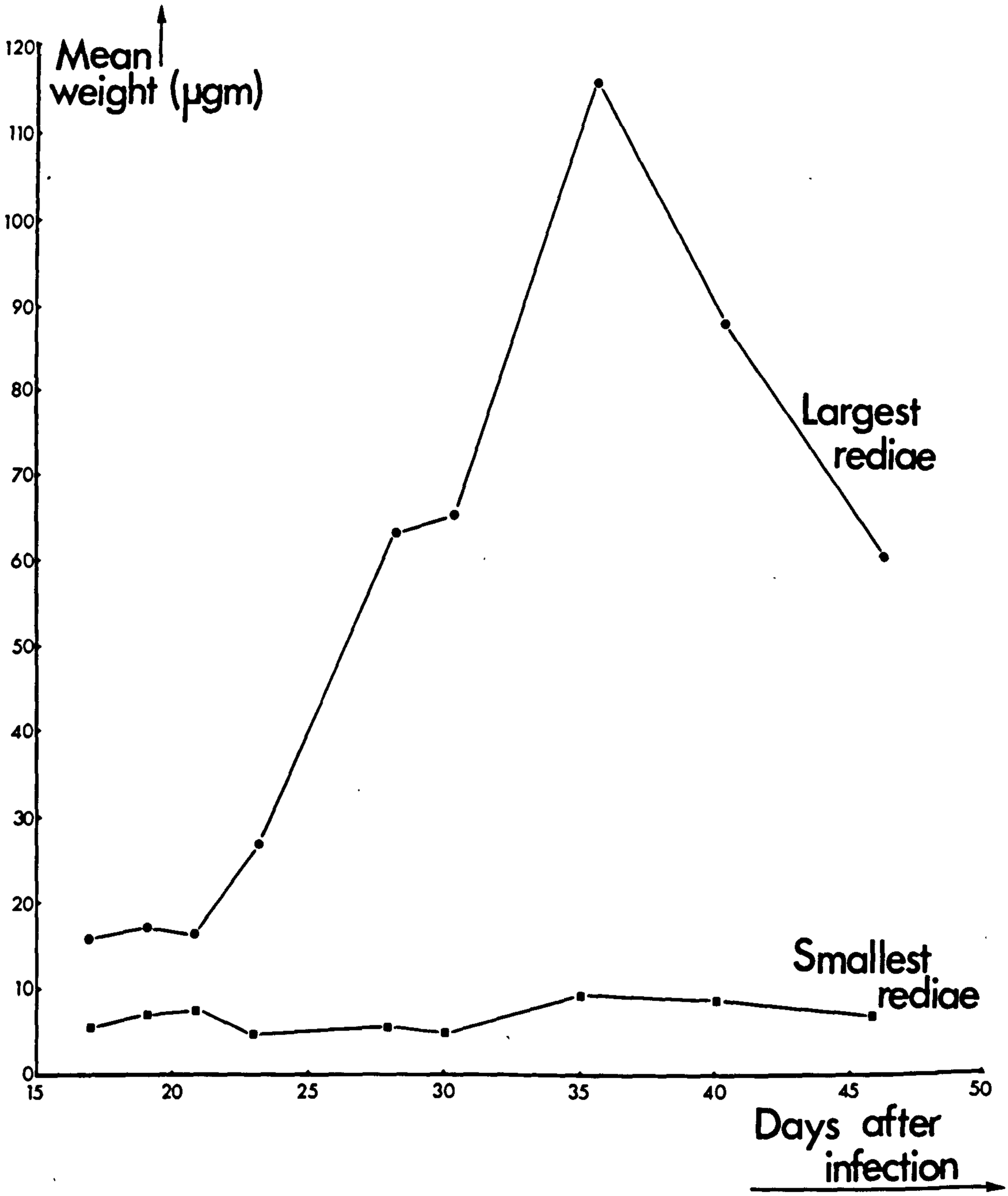
Mean weight (µgm)
120
110
100
90
80
70
60
50
40
30
20
10
0
15
20
25
30
35
40
45
50
Largest rediae
Smallest rediae
Days after infection

FIGURE 17

APPENDIX III

Table 1. - pH measurements

Sample No.	± Centrifuged	Carbon dioxide or nitrogen	pH
1	-	Carbon dioxide	7.48
2	-	"	7.35
3	-	"	7.50
4	-	"	7.48
5	-	"	7.62
6	+	"	7.50
7	+	"	7.60
8	+	"	7.42
9	+	"	7.52
10	+	"	7.68
1	-	Nitrogen	7.84
2	-	"	7.90
3	-	"	8.00
4	-	"	8.08
5	-	"	8.00
6	-	"	8.12
7	-	"	8.16
8	-	"	7.94
9	-	"	8.02
10	-	"	8.10

APPENDIX III

Table 2. - Osmotic pressure measurements.

a) Vapour pressure method: total and % changes in droplet lengths over 7 days (See Figure 2.) Lengths are in arbitrary units to the nearest 0.05 units.

% NaCl	Standard 1.		Sample		Standard 2.		Total % Change
	Change	% Change	Change	% Change	Change	% Change	
1.0	-0.25	7.25	+0.35	8.05	-0.25	5.81	21.11
0.9	-0.10	3.13	+0.20	4.44	-0.25	4.59	12.16
0.8	-0.20	2.88	+0.25	4.63	-0.10	1.37	8.88
0.7	-0.05	0.76	+0.05	0.64	-0.05	0.80	2.20
0.6	-0.05	0.69	+0.05	0.54	-0.05	0.83	2.06
0.5	+0.05	0.69	0	0	+0.05	0.75	1.44
0.4	+0.10	1.64	-0.10	1.80	+0.10	2.44	5.88
0.3	+0.15	2.73	-0.05	0.80	+0.10	1.59	6.12
0.2	+0.20	2.99	-0.15	2.07	+0.30	5.45	10.51
0.1	+0.25	3.88	-0.20	4.80	+0.25	3.47	12.15

b) Melting-points (^{o}C-arbitrary Beckman scale) and Δ for haemolymph and NaCl standards in 1 µl and 5 µl sample tubes.

Tube	Sample	Melting-point	Melting-point of water tube	Δ (^{o}C)
1 µl	1.0% NaCl	2.768	2.180	0.588
1 µl	1.0% NaCl	2.755	2.175	0.580
1 µl	0.5% NaCl	2.480	2.182	0.298
1 µl	Haemolymph	2.404	2.118	0.286
5 µl	Haemolymph	2.398	2.115	0.283

c) Melting-points (^{o}C-arbitrary Beckman scale) and Δ for haemolymph and NaCl standards for the 2 main sets of determinations.

Haemolymph Pool 1	Haemolymph		0.25% NaCl		0.5% NaCl		1.0% NaCl		Water
Determination	M.pt.	Δ	M.pt.	Δ	M.pt.	Δ	M.pt.	Δ	M.pt.
1	2.347	0.279	2.223	0.155	2.365	0.297	2.658	0.590	2.068
2	2.360	0.285	2,244	0.169	2.375	0.300	2.696	0.621	2.075
3	2.449	0.270	2.237	0.158	2.460	0.281	2.738	0.559	2.179
4	2.330	0.280	2.202	0.152	2.348	0.298	2.622	0.572	2.050
5	2.462	0.279	2.332	0.149	2.488	0.305	2.775	0.592	2.183
Haemolymph Pool 2									
1	2.428	0.275	2.324	0.171	2.452	0.299	2.730	0.577	2.153
2	2.382	0.308	2.241	0.167	2.390	0.316	2.647	0.573	2.074
3	2.443	0.279	2.321	0.157	2.451	0.287	2.757	0.593	2.164
4	2.458	0.271	2.352	0.165	2.473	0.285	2.785	0.598	2.187
5	2.426	0.268	2.300	0.142	2.468	0.310	2.805	0.647	2.158

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Table 3. - Solid Content of Haemolymph: Dry Weights of 5µl samples

Sample	Dry Wt. (µg)	Sample	Dry Wt. (µg)
1	92.0	6	106.6
2	89.2	7	88.2
3	81.8	8	95.6
4	95.4	9	110.0
5	84.4	10	97.2

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Table 4. - Sodium and potassium: flame photometric measurements.

a) Sodium calibration

$[Na^+]$ in ppm	Deflections in scale units; 3 sets of readings for each solution.			Mean deflection the plotted value.
	Solution 1.	Solution 2.	Solution 3.	
5	22.5	20.5	22.5	22.1
	22.5	21.5	23.5	
	22.0	20.5	23.5	
10	40.5	39.5	41.5	40.3
	40.0	39.0	41.0	
	39.5	40.0	41.5	
15	53.5	52.5	55.0	53.5
	52.5	52.5	55.0	
	53.0	52.0	55.5	
20	63.5	63.0	66.0	64.2
	64.0	62.5	66.5	
	63.5	62.5	66.5	
25	72.5	71.5	74.5	72.7
	72.0	71.5	74.0	
	72.0	71.5	74.5	
30	80.0	79.0	81.5	80.0
	81.0	78.0	81.5	
	79.5	78.5	81.0	
35	87.0	85.0	87.0	86.4
	86.0	85.5	87.5	
	86.5	85.0	88.0	
40	92.0	91.0	92.5	91.7
	92.5	91.0	92.5	
	92.0	90.5	92.0	
45	97.0	96.0	97.0	
	97.0	95.5	97.0	
	97.5	95.5	97.5	
50 (set)	100 ± 1.5	100 ± 1.5	100 ± 1.5	100

b) Potassium Calibration

$[K^+]$ in ppm.	Deflections in scale units, 6 readings on duplicate solutions		Mean deflection; the plotted value
	Solution 1.	Solution 2.	
1	10.5, 10.5,	10.5, 9.5	10.25
	10.5, 10.0,	10.0,10.0,	
	10.5, 10.5,	11.0, 9.5,	
2	20.0, 20.5,	19.5, 20.0,	20.17
	20.5, 20.5,	20.0,20.0,	
	20.5, 21.0,	20.0,19.5,	
3	32.5, 31.0,	30.0,30.5,	31.21
	33.5, 30.0,	30.0,31.5,	
	33.0, 31.0,	30.0,31.5,	
4	42.0, 42.5,	40.5,41.5,	41.71
	42.5, 42.0,	41.0,41.5,	
	41.5, 43.0,	40.5,42.0,	
5	52.0, 53.0,	50.5,52.0,	52.08
	52.5, 53.0,	51.0,52.5,	
	52.0, 53.0,	51.0,52.5,	
6	62.5, 63.0,	60.5,62.5,	
	62.5, 63.5,	60.5,62.0,	
	62.5, 63.0,	60.0,62.0,	
7	72.5, 73.0,	72.0,71.5	72.08
	73.0, 72.5,	71.0,71.0,	
	72.5, 72.5,	72.0,71.5,	
8	81.0, 82.5,	77.5,80.5,	80.38
	81.0, 81.0,	81.0,77.0,	
	82.5, 82.5,	80.5,77.5,	
9	90.5, 91.0,	88.0,89.5,	89.92
	91.0, 91.5,	87.5,90.0,	
	90.5, 91.0,	88.5,90.0,	
10	100 ± 2.0	100 ± 2.0	100

c) Sodium determinations on haemolymph.

Sample No.	Deflections in scale units, 2 readings per sample.	Mean deflection	"Concentration of Na^+" in ppm from calibration curve.
1	47.5, 48.5	48.00	12.90
2	41.5, 42,5	42.00	10.80
3	43.0, 42.0	42.50	10.95
4	47.0, 46.5	46.75	12.40
5	46.5, 46.0	46.25	12.20
6	45.5, 46.0	45.75	12.10
7	43.5, 43.0	43.25	11.20
8	41.0, 40.0	40.50	10.25
9	38.5, 39.0	38.75	9.70
10	40.5, 39.5	40.00	10.10

d) Potassium determinations on haemolymph.

Sample No.	Deflections in scale units, 2 readings per sample.	Mean deflection	"Concentration of K^+" in ppm from calibration curve
1	14.5, 15.0	14.75	1.38
2	12.5, 12.5	12.50	1.14
3	17.5, 17.0	17.25	1.62
4	16.0, 16.5	16.25	1.53
5	16.5, 17.0	16.75	1.58
6	13.5, 14.0	13.75	1.28
7	16.0, 15.5	15.75	1.48
8	13.0, 12.5	12.75	1.18
9	15.5, 15.5	15.50	1.45
10	16.5, 17.0	16.75	1.58

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Table 5. - Calcium and magnesium determinations.

a) First Calcium calibration.

"Concentration of Ca^{++}" in ppm	Deflections in scale units; triplicate solutions with one reading each.			Mean deflection (plotted value)
	Solution 1.	Solution 2.	Solution 3.	
1	0.49	0.51	0.51	0.50
2	0.95	0.97	0.96	0.96
3	1.43	1.40	1.45	1.43
5	2.25	2.28	2.29	2.27
10	4.24	4.32	4.30	4.29

b) Second calcium calibration: containing trichloroacetic acid and Sr^{++}

"Concentration of Ca^{++}" in ppm	Deflections in scale units; triplicate solutions, with one reading each.			Mean deflection (plotted value)
	Solution 1.	Solution 2.	Solution 3.	
1	0.55	0.56	0.56	0.56
2	1.02	1.04	1.05	1.03
3	1.58	1.56	1.57	1.57
5	2.49	2.47	2.50	2.49
10	4.40	4.38	4.39	4.39

c) Third and fourth calcium calibrations and concurrent haemolymph determinations.

Sample	Deflection ± 0.025 due to instrumental instability
1 ppm Ca^{++}	0.50
3 "	1.38
5 "	2.18
7 "	3.06
10 "	4.15
Haemolymph.1.	0.62
Haemolymph.2.	0.64
Haemolymph.3.	0.88
1 ppm Ca^{++}	0.51
3 "	1.35
5 "	2.18
7 "	3.02
10 "	4.00
Haemolymph 4.	0.92
Haemolymph 5.	0.60
Haemolymph 6.	0.85

d) First Magnesium calibration.

"Concentration of Mg^{++}" in ppm	Deflection in scale units.
1	1.46
2	2.88
3	4.05

e) Second magnesium calibration and concurrent haemolymph determinations

Sample	Deflection ± 0.025 due to instrumental instability
0.5 ppm Mg^{++}	1.68
1.0 ppm Mg^{++}	3.20
2.0 ppm Mg^{++}	5.98
Haemolymph 1.	1.62
Haemolymph 2.	1.67
Haemolymph 3.	1.68
Haemolymph 4.	1.60
Haemolymph 5.	1.64
Haemolymph 6.	1.70

APPENDIX III

Table 6. - Chloride titrations (all values in µl.)

a) Determinations of chemical control serum using approx. 0.1N mercuric nitrate.

Blank titrations (distilled water + reagents)		50 mEq/litre Cl^- standard		Control serum	
Rough.	0.1	Rough.	10.5	Rough.	20.3
Accurate	0.56	Accurate	10.403	Accurate.	20.115
	0.060		10.412		20.195
	0.050		10.425		20.130
	0.074		10.422		20.137
	0.075		10.418		20.152
Mean =	0.063	Mean =	10.416	Mean =	20.146

b) Determinations on test saline using approx. 0.05N mercuric nitrate

Blank titrations (distilled water + reagents)		50 mEq/litre Cl^- standard		Test saline	
Rough.	0.3	Rough.	21.0	Rough.	22.70
Accurate	0.254	Accurate.	20.842	Accurate.	22.372
	0.218		20.883		22.414
	0.244		20.846		22.460
	0.210		20.838		22.352
	0.268		20.856		22.405
Mean =	0.239	Mean =	20.853	Mean =	22.405

c) Determination on haemolymph using approx. 0.05N mercuric nitrate

Blank titrations (distilled water + reagents)	50 mEq/litre Cl^- standard	Haemolymph	
as b.) above	as b.) above	12.870	
		13.689	old sample
		12.062	
		13.426	
		13.515	
		13.560	
		13.502	Fresh samples
		13.452	
	Mean =	13.491	

Table 7. - Bicarbonate determinations/conductivity measurements.

a.)

Temperature (^{o}C)	Conductivity (mho. x 10^{-2})*
20.4	0.495
21.0	0.500
23.0	0.511
23.8	0.518
25.3	0.535
29.1	0.555

b.) Changes in conductivity observed for bicarbonate standards and haemolymph samples

Solution		Change in Conductivity*		Ambient Temperature (^{o}C)		
250 ppm HCO_3^-	Sample 1	0.018	Initial	16.2	Final	16.3
	Sample 2	0.017		16.3		16.4
	Sample 3	0.019		16.6		16.4
500 ppm HCO_3^-	Sample 1	0.040	Initial	19.6	Final	19.4
	Sample 2	0.038		19.2		19.2
	Sample 3	0.038		19.3		19.6
1000 ppm HCO_3^-	Sample 1	0.072	Initial	18.0	Final	18.3
	Sample 2	0.073		18.3		18.3
	Sample 3	0.073		18.2		18.6
1500 ppm HCO_3^-	Sample 1	0.108	Initial	19.6	Final	20.0
	Sample 2	0.104		16.3		16.4
	Sample 3	0.110		16.4		16.4

Solution	Change in conductivity*	Ambient Temperature (^{o}C)	
Haemolymph	Sample 1 0.078	Initial 19.6	Final 19.4
	Sample 2 0.083	19.0	19.2
	Sample 3 0.079	18.8	18.7
	Sample 4 0.084	18.6	18.6

*(Measurement made on the 10^{-2} scale of the Pye 11700 Bridge)

APPENDIX III

Table 8. - Phosphate determinations

a) Free phosphate; optical densities for phosphorus standards.*1.

"Concentration of Element P" (ppm)	Optical densities (duplicate tubes 2 readings for each)		Mean O.D.
	I	II	
5	0.050, 0.051	0.050, 0.050	0.050
10	0.105, 0.102	0.109, 0.108	0.106
15	0.149, 0.148	0.152, 0.150	0.150
20	0.201, 0.198	0.206, 0.207	0.203
50	0.521, 0.522	0.524, 0.522	0.522

*1. Read against pooled blanks. The difference between blanks before pooling was ± 0.003.

b) Free phosphate; optical densities for haemolymph samples and concurrent phosphorus standards.*2.

Sample	Optical densities (duplicate tubes, 2 readings for each)		Mean O.D.
	I	II	
Haemolymph 1.	0.022, 0.023	0.028, 0.028	0.025
Haemolymph 2.	0.025, 0.026	0.030, 0.029	0.028
10 ppm P.	0.112, 0.110	0.108, 0.107	0.109
20 ppm P.	0.208, 0.210	0.214, 0.212	0.211
5 ppm P.	0.052, 0.052	0.054, 0.053	0.053

*2. Read against pooled blanks. The difference between blanks before pooling was ± 0.002. Haemolymph 1. was run with the 10 and 20 ppm standards, and Haemolymph 2. with the 5 ppm standard.

c) Total phosphate; optical densities for phosphorus standards (first run).*3.

"Concentration of Element P" (ppm)	Optical density (pooled duplicates)
5	0.055
10	0.134
15	0.212
20	0.261
50	0.568

*3. Read against a single pooled blank.

d) Total phosphate; optical densities for phosphorus standards (second run)*4.

"Concentration of Element P" (ppm)	Optical densities (triplicate tubes 3 readings for each)			Mean O.D.
	I	II	III	
5	0.042, 0.041	0.062, 0.062	0.045, 0.046	0.050
10	0.097, 0.097	0.086, 0.085	0.093, 0.094	0.092
15	0.143, 0.142	0.151, 0.151	0.145, 0.146	0.146
20	0.018, 0.181	0.174, 0.174	0.179, 0.178	0.178
50	0.433, 0.432	0.448, 0.448	0.454, 0.455	0.445

*4. Read against pooled blanks. The difference between blanks before pooling was $\pm$ 0.004.

e) Total phosphate; optical densities for haemolymph samples and concurrent phosphorus standards. *5

Sample	Optical densities (triplicate tubes, 2 readings for each).			Mean O.D.
	I	II	III	
Haemolymph 1.	0.026, 0.027	0.030, 0.030	0.019, 0.020	0.025
5 ppm. P.	0.048, 0.048	0.041, 0.040	0.040, 0.039	0.044
15 ppm. P.	0.133, 0.132	0.124, 0.124	0.127, 0.127	0.128
50 ppm. P.	0.436, 0.435	0.442, 0.442	0.433, 0.433	0.437
Haemolymph 2.	0.036, 0.035	0.029, 0.030	0.033, 0.033	0.033
2.5 ppm. P.	0.018, 0.018	0.024, 0.023	0.025, 0.025	0.022
5.0 ppm. P.	0.044, 0.043	0.049, 0.050	0.038, 0.039	0.044

*5 Read against pooled blanks. The difference between blanks before pooling was ± 0.003 for the first set, and ± 0.001 for the second set.

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Table 9. - Glucose determinations

a) Volumes of reagents for colour development.

Tube	distilled-deionised water	0.01% glucose standard	haemolymph supernatant	copper reagent	arseno-molybdate reagent
Blank	100 µl	-	-	100 µl	100 µl
Standard	-	100 µl	-	100 µl	100 µl
Haemolymph	-	-	100 µl	100 µl	100 µl

b) Optical densities read for haemolymph samples and concurrent standards.*1

Sample	Optical densities (triplicate standards, four haemolymph samples)				Mean O.D.
	I	II	III	IV	
Haemolymph	0.245	0.229	0.230	0.240	0.236
0.01% glucose standard.	0.143	0.148	0.144		0.145

*1 Read against pooled blanks. The difference between blanks before pooling was ± 0.004.

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Table 10 - Nitrogen determinations

a) Total nitrogen; optical densities read for the first calibration attempt using triplicate standards.*1

Nitrogen (µg)	Optical densities			Mean O.D.
	I	II	III	
23.68	0.532	0.545	lost	0.539
35.52	0.772	0.774	lost	0.773
47.36	1.114	1.232	1.280	1.213
59.20	1.430	1.495	1.346	1.413

*1 Read against pooled duplicate blanks (one was lost). The difference between the blanks before pooling was ± 0.009. Added NaOH was 8.3 ml.

b) Total nitrogen; optical densities read for the second calibration and for 6 haemolymph samples.*2

Sample	Optical densities			Mean O.D.
	I	II	III	
11.84 µg N.	0.266	0.280	0.295	0.280
23.68 µg N.	0.551	0.543	0.577	0.570
59.20 µg N.	1.444	1.419	1.431	1.431
Haemolymph	0.312	0.325	0.314	0.320
Haemolymph	0.310	0.342	0.318	0.323

*2 Read against pooled blanks. The difference between blanks before pooling was ± 0.005. Added NaOH was 8.2 ml.

c) Non-protein nitrogen; optical densities read for nitrogen standards and 5 haemolymph samples.*3

Sample	Optical densities			Mean O.D.
	I	II	III	
4.5 µg N.	0.145	0.143	0.158	0.149
9.0 µg N.	0.287	0.325	0.316	0.309
Haemolymph	0.088	0.099	0.102	0.097
	0.096	0.100		
3.9 µg N.	0.138	0.118	0.124	0.130
	0.129	0.139		

*3 Read against pooled blanks. The difference between blanks before pooling was ± 0.005. Added NaOH was 2.0 ml.

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Table 11 - Results of amino acid chromatograms. ('+' indicates a compound identified but not determined quantitatively)

a) Calibration run using a Technicon standard mixture (0.01 μMoles of each amino acid)

Compound	Area of peak	Comments
Aspartic acid	8.946	Well-defined peak.
Threonine	36%) Trapezoidal area = 1.063	Peaks partially merged.
Serine	64%)	
Glutamic acid	8.797	Well-defined peak.
Proline	0.276	Small peak, not reliable.
Glycine	10.240	Well-defined peak.
Alanine	9.690	"
Valine	10.320	"
Cystine	5.200	"
Methionine	10.860	"
Isoleucine	12.210	"
Leucine	12.360	"
Tyrosine	11.490	"
Phenylalanine	+	Ammonia rise stopped integration.
Ammonia	0.324	Integrating the base line peak.
Lysine	+	Ammonia rise stopped integration.
Ornithine	+	"
Histidine	+	"
Arginine	NOT FOUND	Arginine WAS in the mixture.

b) Chromatogram from the first haemolymph sample (40 µ l.)

Compound	Area of peak	Comments
Cysteic acid	0.600	Small peak, possibly a breakdown product of Cystine.
Hydroxy-L-proline	+	Small peak merged.
Aspartic acid	+	with Aspartic acid.
Threonine	+	Recorder jammed.
Serine	+	"
Glutamic acid	6.050	Well defined peak.
Proline	0.748	Small peak, not reliable.
Glycine	5.250	Well-defined peak.
Alanine	19.720	"
∝ -amino-n-butyric acid	0.896	Small peak.
Valine	8.620	Well-defined peak.
Cystine	0.440	Small peak.
Methionine	2.244	Well-defined peak.
Isoleucine	3.652	"
Leucine	4.848	"
Nor-Leucine	15.023	"
Tyrosine	+	Ammonia rise stopped integration.
Phenylalanine	+	"
Ammonia	3.074	Integrating the base line peak.
Ornithine	+	Ammonia rise stopped integration.
Lysine	+	"
Histidine	+	"

c) Chromatogram from the second haemolymph sample (50 µl).

Compound	Area of peak	Comments
Cysteic acid	0.640	as b.
Hydroxy-L-proline	+	as b.
Aspartic acid	1.836	Better separation than b.
Threonine	33.4%) Trapezoidal	Peaks merged, but areas can be calculated.
Serine	66.6%) = 0.887 area	
Glutamic acid	5.858	as b.
Proline	0.800	"
Glycine	6.844	"
Alanine	20.709	"
∝-amino-n-butyric acid	+	Very small peak.
Valine	7.114	as b.
Cystine	0.672	"
Methionine	1.800	"
Isoleucine	3.450	"
Leucine	5.335	"
Nor-Leucine	16.650	"
Tyrosine	+	"
Phenylalanine	+	"
Ammonia	3.990	"
Ornithine	+	"
Lysine	+	as b.
Histidine	+	"

d) Chromatogram from the third haemolymph sample (50 µl)

Compound	Recorder expansion	Area of peak	Comments
Cysteic acid	X4	0.510	as b.
Hydroxy-L-proline	X4	+	as b.
Aspartic acid	X10	+	Off scale.
Threonine	X10	+	"
Serine	X10	+	"
Glutamic acid	-	-	lost while changing colorimeter leads.
Proline	X4	2.444	Colorimeter leads changed.
Glycine	X4	6.188	as b.
Alanine	X4	21.190	"
α-amino-n-butyric acid	X10	1.68	"
Valine	X10	30.626	"
Cystine	-	-	Lost while changing expansion.
Methionine	-	-	"
Isoleucine	X4	3.364	as b.
Leucine	X4	4.192	"
Tyrosine	X4	+	"
Phenylalanine	X4	+	"
Ammonia	X4	7.276	"

APPENDIX III

Table 12 - Total protein determinations

a) Optical densities read for protein standards.*1

Protein (µg)	Optical densities (duplicates)		Mean O.D.
	I	II	
35	0.068	0.075	0.072
70	0.134	0.132	0.133
105	0.183	0.189	0.186
140	0.238	0.232	0.235
210	0.305	0.298	0.302

*1 Read against pooled blanks. The difference between blanks before pooling was ± 0.005.

b) Optical densities read for haemolymph samples and concurrent standards.*2

Sample	Optical densities (duplicate standards, 3 and 4 haemolymph samples).		Mean O.D.
	I	II	
5µl Haemolymph	0.110	0.118	0.114
10µl Haemolymph	0.220		0.220
70µg Protein	0.120	0.122	0.121
140µg Protein	0.218	0.213	0.216
5µl Haemolymph	0.107 0.107	0.108 0.105	0.107
70µg Protein	0.116	0.121	0.185
140µg Protein	0.214	0.222	0.218

*2 Read against pooled blanks. For both sets of determinations the difference between blanks before pooling was ± 0.004.

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Table 13 - Cholesterol determinations

Sample	Optical densities (triplicate standards, 5 haemolymph samples)			Mean O.D.
	I	II	III	
10 µg cholesterol	0.028	0.029	0.027	0.028
20 µg cholesterol	0.059	0.058	0.057	0.058
40 µg cholesterol	0.120	0.122	0.124	0.122
Haemolymph	0.069	0.044	0.050	
	0.048	0.059		

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